Factors influencing the behaviour of Salmonella spp. in the hen's egg

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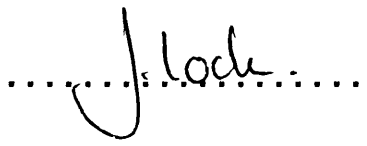

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SUMMARY

This study identified in greater detail the events following bacterial contamination of the shell membranes or albumen of the hen's egg. The course of salmonella infection of whole eggs was the same as that in eggs infected with rot producing bacteria. Thus there was a lag of 10-20 days between infection of whole eggs and the onset of gross contamination of the albumen with storage. The results corroborated existing evidence that ovotransferrin is the principal member of the chemically based antimicrobial defence of eggs. Thus the bacteriostatic action of albumen against salmonellas was negated by the addition of Fe^{3+} in concentrations sufficient to saturate the chelating potential of ovotransferrin. This response was a feature of cells that were physiologically fit at the time of inoculation of albumen as well as those subjected to temperature or pH stress before inoculation.

It was shown that salmonellas present in an excised inner air cell membrane suspended in albumen alone remained quiescent, but a proportion remained motile until the chelating potential of ovotransferrin was quenched with Fe^{3+}. With infected membranes suspended in broken out whole eggs, the quiescent state persisted until organisms made contact with the yolk. These studies provided for the first time evidence that chemotaxis plays a role in the movement of bacteria from the initial site of infection to the yolk surface. *Pseudomonas putida* was used to study this phenomenon in greater detail because the production of a fluorescent pigment occurred only when the cells were

actively multiplying. It was demonstrated that growth and fluorescence production was initiated by contaminants that had migrated to the surface of the yolk. Thereafter there was a progressive outward movement of fluorescent pigment until eventually all of the albumen fluoresced. This phase of the study provided for the first time compelling evidence that the viscosity of the albuminous sac provided a physical constraint to the movement of bacterial contaminants of the albumen both towards and away from the yolk.

INTRODUCTION

There is a long history of association (Table 1) between poultry, eggs, various serotypes of *Salmonella* and human salmonellosis (Rubenstein, Feemster and Smith 1944). This fact has been highlighted by the recent episodes of salmonellosis in which *S.enteritidis* and other serotypes have been associated with the consumption of products containing raw shell eggs (Table 1), particularly homemade mayonnaise (Perales and Audicana 1989, Perales and Garcia 1990), ice-cream and shop-bought sandwiches containing mayonnaise (Cowden *et al.* 1989). Indeed the past five years have seen a dramatic increase in the number of outbreaks of food poisoning attributable to this organism. A World Health Organization surveillance report noted a median increase of 126% in 24 of the 35 countries, including eight European ones, studied between 1979 and 1987 (Rodrigue, Tauxe and Rowe 1990). This trend was evident in England and Wales also (Cowden *et al.* 1989). There were 14,000 cases of *S.enteritidis* in 1988, two-thirds of which were due to Phage Type 4 (Figure 1). This serotype may well have affected up to 2 million people in the United Kingdom (Sharp 1989). In a 2-year period before 1988 this organism had been associated mainly with holiday makers returning from southern European countries (Sharp 1989). Previous to the current outbreaks, *S.enteritidis* had been associated with salmonellosis due to the consumption of duck eggs (Wilson 1948). The north-eastern United States witnessed a 6-fold increase in the number of cases of

human salmonellosis caused by other phage types - PT 8 and PT 13a - of *S.enteritidis* between 1976 and 1986. A total of 2119 cases and 11 deaths were recorded between January 1985 - May 1987. Seventy seven percent of these outbreaks were traced to Grade A shell eggs or foods made from such eggs (St Louis *et al.* 1988). Before 1976 *S.enteritidis* accounted for only 6% of food poisoning outbreaks in the USA (Madden 1990).

Although there is a long history of eggs being implicated in human salmonellosis (Scott 1930), the literature does not identify precisely the infection route(s) of eggs. Some consider that *Salmonella* are transported to the yolk whilst it is being formed in the ovary (Rettger 1914). Others favour the view that infection occurs after laying *via* the pores in the egg shell (Stokes, Osborne and Bayne 1956). Indeed the latter contention led to the belief that an egg's susceptibility to infection post oviposition was linked to shell porosity. This attribute had a nebulous definition until the classic studies of Rahn and his collaborators (1979) who described porosity in its strict biological context, namely the shell's potential for the diffusion of respiratory gases. Later studies (Humphrey *et al.* 1991) have provided evidence that oviducal infection is probably the main route of egg contamination, with salmonellas lodged mainly in the albumen. The causes of oviduct infection, however, have not been determined. In practice the infection of the albumen and perhaps also the shell membranes rather than

the yolk means that the behaviour of *Salmonella* in eggs infected oviducally may well mimic that of spoilage organisms which infect eggs *via* the pores in the shell. The latter topic has been investigated over the past 100 years (Haines 1939, Board and Fuller 1974). Indeed Clay and Board (1991) presented evidence in support of this contention and circumstantial evidence that the initial salmonella contaminants of the albumen remained quiescent until some event, probably contact with the yolk, induced growth. Thus, the literature on the addling of eggs (Haines 1939) and the study by Clay and Board (1991) leave the impression that the contaminants of the albumen are passive at the outset of the process leading to a generalized infection of an egg's contents. Those that initiate the latter do so by chance contact with the yolk. Recently Humphrey (1991) contended that changes in the chemical composition of the albumen play a part in triggering the growth of contaminants. The present study sought to reconcile these contentious views on the events leading to generalized infection of the hen's egg with salmonellas. In practice answers to the following questions were sought. (1). What factors influence the behaviour of *S.enteritidis* and other serotypes in albumen *in vitro* ? (2). Do somnicells behave in the same manner as young cells of *S.enteritidis* in albumen ? (3). Does chemotaxis play a role in the events culminating in generalized egg infection ?

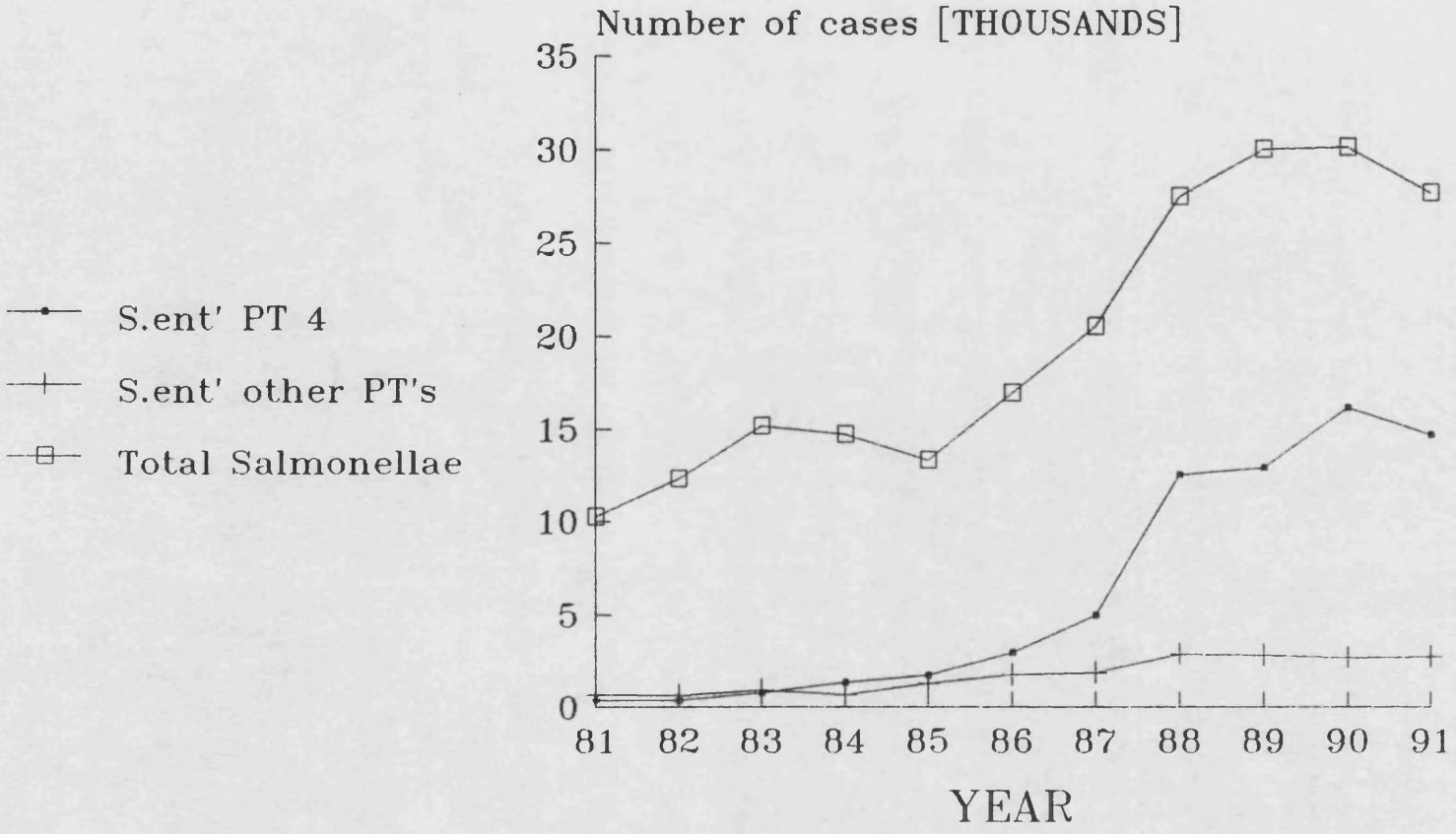
Table 1. Egg products implicated in salmonellosis

Food Source	*Salmonella* serotype	Reference
Mayonnaise	*enteritidis*	Cowden *et al.* (1989) Telzak *et al.* (1990) North & Gorman (1990)
	typhimurium	Mitchell *et al.*(1989) Stevens *et al.* (1989) Greenblatt *et al.*(1946)
Hollandaise Sauce	*enteritidis*	Guzewich (1988) St Louis *et al.* (1988) Stevens *et al.* (1988) Morris (1990) Mishu *et al.* (1991)
Egg Nog	*enteritidis*	Guzewich (1988) Anon (1987) St Louis *et al.* (1988)
	typhimurium	Philbrook *et al.* (1960) Ager *et al.* (1967) Steere *et al.* (1975)
Egg Whip	*thompson*	Knowles (1971) Broomhead & Mann (1959)
Mousse	*enteritidis*	Anon (1988) Mawer *et al.* (1989)
Egg Salad	*montevideo*	Watt (1945)
Caesar Salad	*enteritidis*	Anon (1987)
Sandwiches	*enteritidis*	Perez *et al.* (1986) Anon (1987) Guzewich (1988) St Louis *et al.* (1988) Morris (1990) Coyle *et al.* (1988)
Rice Balls	*enteritidis*	Anon (1988) Guzewich (1988) St Louis *et al.* (1988)
Scotch Eggs	*enteritidis*	Coyle *et al.* (1988)
Savoury Quiche	*typhimurium*	Anon (1987)
Eggs:Scrambled	*derby*	Ager *et al.* (1967)
	enteritidis	Anon (1987)
Fried	*enteritidis*	Lin *et al.* (1988)
Omelettes	*enteritidis*	Anon (1988) Guzewich (1988) St Louis *et al.* (1988) Coyle *et al.* (1988) Morris (1990)

Pasta	*enteritidis*	Anon (1987) Guzewich (1988) St Louis *et al.* (1988)
Ice Cream	*enteritidis*	Anon (1988) Coyle *et al.* (1988) Cowden *et al.* (1989) Palmer & Coyle (1989)
	typhimurium	Gunn & MarkaKis (1978)
	typhimurium/ branderup	Armstrong *et al.*(1970)
Meringue	*typhimurium*	Anon (1987)
	heidelberg	Ager *et al.* (1967)
Cakes	*paratyphi B*	Knowles (1971)
	thompson	Knowles (1971) Bowmer (1965)
	typhimurium	Chapman *et al.* (1988) Anon (1987)
	enteritidis	Cowden *et al.* (1989) Morris (1990)
	heidelberg	Bowmer (1965)
Cake Filling	*enteritidis*	St Louis *et al.* (1988)
Custard, Trifle Cream Confectionery	*thompson* *typhimurium* *paratyphi B* *oranienberg* *infantis* *scwarzengrund* *enteritidis* *orion* *thompson* *typhimurium*	Anderson *et al.* (1935) Harvey & Price (1961) Newell *et al.* (1955) Knowles (1971)
Milk Drinks	*enteritidis*	Cowden *et al.* (1989)
Tapioca Pudding	*heidelberg*	Ager *et al.* (1967)

Figure 1

Cases of *Salmonella* food poisoning in England and Wales 1981–1991



S.ent' = Salmonella enteritidis

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LITERATURE REVIEW

The mode of bacterial infection of the hen's egg attracted renewed attention once the egg was implicated in the transmission of *S.enteritidis* to man in the recent outbreaks. Infection of the laying hen - and hence eggs - may be the consequence of horizontal or vertical transmission. The former will be considered in the first instance. In this case transfer of salmonellas occurs from bird-to-bird, man-to-bird, feed-to-bird or environment-to-bird (Madden 1990). Thus salmonella-free birds may become infected by contact with any infected source in the environment. Studies of this aspect with one-day old chicks inoculated orally (Williams Smith and Tucker 1980) showed that the percentage of chickens infected was dependent upon the organisms to which they were exposed. Thus, 68% of birds became infected on exposure to *S.menston*; 48% to *S.agona*; 32% to *S.typhimurium* PT 36; 28% to *S.enteritidis*; and 12% to *S.senftenberg*.

The extensive review by Williams (1981) of a very large number of studies leaves little doubt that feed is the principal source of *Salmonella* contamination in horizontal transmission in hens. This contention received support from the recent work of Jones *et al.* (1991) who collected material from feed mills and broiler/multiplier houses in the USA. They found that 20.8% of feed samples were contaminated with various serotypes of *Salmonella*. The individual ingredients of the feed samples were contaminated at the following

rates: bone meal, 60%; poultry meal, 40%; fish meal, 33%, and other ingredients, 20.8%. *Salmonella typhimurium* was isolated most frequently, but 22 other serotypes including *S.agona, S.anatum, S.hadar, S.infantis* and *S.thompson* were isolated also. Similar incidences of salmonella contamination of bone meal were noted by Morris *et al.* (1969). In hatcheries, 9.6% of the yolk sacs from one-day old chicks were infected with *S.typhimurium.* As this organism was the commonest contaminant of feed, it was probable that the latter was the source of contamination at the broiler/multiplier farm. *Salmonella enteritidis* was not found at the feed mill, the hatchery or in the multiplier houses (Jones *et al.* 1991). A Canadian study isolated salmonellas from 13.4% of the samples of feed supplied to 300 flocks. The same serotypes were isolated subsequently from water and litter taken from the poultry houses (Poppe *et al.* 1991). *Salmonella hadar* was isolated most frequently. *Salmonella enteritidis* was isolated from only 3.1% of flocks and never from feed.

The increasing number of outbreaks of salmonellosis in humans led to the examination of ovaries (3,600) and intestines (1,200) from 14 American commercial flocks. The causative organism, *S.enteritidis*, was not isolated (Baker, Goff and Timoney 1980). Barnhart *et al.* (1991) collected ovaries from 42 commercial layer flocks at slaughter. Seventy-six percent of the flocks were infected at a rate of >10%, with *S.heidelberg* being the predominant serotype (56.5%). *Salmonella enteritidis* PT

23 was recovered from one flock only. It was noted, however, that 6 of the 10 serotypes from human and non-human sources most frequently reported by the CDC were detected in the ovaries included in this study. In a similar study by Jones *et al.* (1991), 69 out of a total of 101 ovaries obtained from a breeder flock contained *S.typhimurium.* Following a salmonella outbreak in a New York hospital, an examination of 555 hens' ovaries revealed that 69% harboured salmonellas. It was noted, however, that contamination occurring at the processing plant may have been responsible for this heavy incidence of infection (Benson and Eckroade 1991). *Salmonella enteritidis* PT 4 has been found to be a common contaminant of poultry in England and Wales, particularly of the ovaries and oviduct (Anon 1989).

Salmonella enteritidis is an invasive strain capable of invading the digestive system and blood stream of the bird (Hinton *et al.* 1989). *Salmonella enteritidis* PT 4 has been shown to be more invasive for young chicks than PTs 7, 8 and 13a when included in feed. Phage type 4 was isolated from 96% of caecal contents and 27-100% of livers in infected birds (Hinton, Threlfall and Rowe 1990). Similar studies by Barrow (1991) showed that *S.enteritidis* PT 4, 6 and 8 were more virulent than PT 13a (a UK isolate) for newly hatched chicks infected orally. It was noted also that PT 4 and 6 were isolated from the spleen 12 hours post-inoculation; PT's 8 and 13a were not. After 24 hours the numbers of PT 4 isolated from the spleen were 10 times those of PT 13a. These

studies provide ample evidence of a link between the carriage of salmonellas by laying birds and their occurrence in human disease.

Should a laying hen become a carrier of salmonellas, there are 3 possible routes of egg infection: transovarian, oviducal or trans-shell (Duguid and North 1991). The first 2 of these would be of immediate importance in vertical transmission.

Transovarian infection of the egg

A chick hatched from a contaminated egg may harbour *Salmonella* in its internal organs for 12-18 months (Anon 1973). The organism may be excreted intermittently in the faeces or in eggs once a female reaches sexual maturity. This could cause the cycle of infection to be continued from one generation to the next (Anon 1973).

During an egg's formation the yolk may become infected whilst still attached to the ovary (Duguid and North 1991). The yolk components are synthesized in the liver and transported *via* the blood to the follicular walls of the ovary where the oocyte develops. Two stages are recognised, an initial slow stage lasting 60 days, and a rapid one during which most of the yolk is deposited. The latter continues until 24 hours before an ovum is released into the oviduct. In practice, therefore, there is a long time span during which the yolk could become infected with salmonellas.

O'Brien (1990) recovered *S.enteritidis* PT 4 from the yolk sacs of chickens hatched from the eggs of

infected grandparent stock. The same phage type was isolated from the progeny of 12 different parent flocks owned by 3 separate organizations. This indicated widespread transmission by the grandparents with possible transovarian infection being the root cause. As such transfer occurs without the bird showing symptoms (Taylor 1969), it is difficult to detect routinely. Indeed Barnhart *et al.* (1991) demonstrated that birds with infected ovaries remained normal with regard to behaviour, egg production and mortality rates, and had no lesions in their visceral organs. These results are in agreement with those of Brown, Ross and Smith (1976), Hopper and Mawer (1988), Jones *et al.* (1991) and Salvat *et al.* (1991). Indeed the last mentioned highlighted the problem of birds which do not excrete *S.enteritidis* but harbour the organism in tissues such as the spleen and ovaries. It is these birds which are potentially the most dangerous with regard to the use of their eggs, and the possibility of food poisoning in man.

Heavily contaminated ovaries may become inactivated in the sense that yolks fail to form (Lister 1988). Infection of the bird does not always lead to *Salmonella* infection of the ovaries (Horox 1989), perhaps because of the serotype involved. This was confirmed in a study of point-of-lay pullets infected orally with *S.infantis*. Infection was produced in some of the body tissues including the digestive system, but not the ovaries (Brown, Ross and Smith 1976).

Salmonella enteritidis PT 4 has surface antigens in common with the avian adapted *S.pullorum*, an organism widely noted for its ability to cause ovule infection (Kampelmacher 1963, Snoeyenbos, Smyser and Van Roekel 1969). The former also possesses the same virulence gene regions as *S.typhimurium* (Pohl *et al.* 1991). These factors may account for the invasiveness noted by Hinton *et al.* (1990), and the sucessful adaptation to and establishment of *S.enteritidis* in British poultry flocks (Rampling *et al.* 1989). Food and water deprivation may also increase the level of salmonella colonization of hens (Mishu *et al.* 1991). He noted that a flock implicated in an outbreak of *S.enteritidis* in man had been deprived of water shortly before the outbreak occurred.

The available evidence relating to transovarian infection suggests the following.

(1). The establishment of *S.enteritidis* infection is dependent upon the age of the bird. Young chicks (1-14 days) are more susceptible to infection with *Salmonella* than young pullets and hens. Chicks rapidly become resistant to infection by food poisoning strains. For example an oral dose of *S.typhimurium* killed 79% of one-day old chicks, but only 3% of 2-day old ones (Williams Smith and Tucker 1980). Younger birds (<20 weeks) are more resistant to infection than older ones (52 weeks), and they are often able to eliminate infection by 16-22 weeks of age (Snoeyenbos *et al.* 1969). Older birds may be more susceptible to infection because of age-induced

changes in their immune systems. Calcium depletion caused by intensive laying may be a contributory factor (Humphrey *et al.* 1991). Low calcium levels reduce the rate of secretion of gonadotrophin. This reduces the rate of follicle growth and therefore the rate of ovulation (Taylor 1966) and may even favour bacterial colonization of the oviduct by removing protection conferred by the secretion of albumen. The protection conferred on younger birds by oestrogen may also have a beneficial effect (Humphrey *et al.* 1991). Bolder, van Lith and Mulder (1991), however, state that there is no physiological reason to account for differences in the susceptibility of hens of any age to *S.enteritidis* infection. Small numbers of contaminated eggs are laid over a short period of time in younger birds, but a much longer time span in older ones (Gast and Beard 1990).

(2). An ovary in an infected hen commonly contains many normal ovules the incidence of which is much greater than that of contaminated ones (Rettger 1914, Hopper and Mawer 1988).

(3). *Salmonella* are present in low numbers in contaminated eggs (Rettger 1914, Hopper and Mawer 1988, Humphrey 1991) and, on the majority of occasions, are isolated from the albumen or "whole" yolk but not the yolk contents (thus implying infection of the vitelline membrane - Humphrey 1991, Benson and Eckroade 1991). Analogous observations were made by Bale and Hinton (1991-unpublished) who inoculated pullets either orally

or cloacally and examined the birds and eggs for infection. Ovaries and oviducts were contaminated with *Salmonella* in all groups of cloacally infected birds. These organs were not found to be contaminated in birds inoculated orally. The available evidence (Table 2) indicates a very low frequency of egg contamination occurring on or in close proximity to the yolk (Anon 1989, Kampelmacher 1963). For example, a study of 5,700 eggs from 15 naturally infected flocks showed that the contents of 0.6% of eggs were contaminated with very low numbers (<10 salmonellas/egg) of *S.enteritidis*. It was deduced that the majority of eggs harboured the organism in the albumen and not the yolk (Humphrey *et al.* 1991). This evidence supports the view that contamination occurs most commonly in the yolk/vitelline membrane and/or albumen whilst the yolk is descending the oviduct (Gast and Beard 1990). This evidence would lead one to question the emphasis given to transovarian infection in hens. In the evidence on salmonella contamination of eggs presented by Humphrey *et al.* (1991), it was notable that contaminated eggs appeared to occur in clusters. There was no evident periodicity about the clustering and to date no adequate explanation has been offered.

It is tempting to speculate that infection of the egg contents may result from gross disturbance of the hen during egg formation a view discussed by Mishu *et al.* (1991). Stress due to "fright" in hens has been linked with certain abnormalties of eggs. Thus at one time it was contended that violent disturbance caused damage to

Table 2. Transovarian/oviducal infection of the contents of hens eggs

Serotype (dose/bird)	Route*	No. eggs contaminated (level of contamination)	Reference
Deliberate Infection			
pullorum (6.2×10^5)	Ovary	6/11 (0.9-4.6/g)	Forsythe *et al.* (1967)
anatum (7.0×10^3)	Ovary	0/9	Forsythe *et al.* (1967)
typhimurium (10^6-10^9)	Oral	0/226	Baker *et al.* (1980)
senftenberg (10^6)	Oral	0/79	Cox *et al.* (1973)
thompson (10^6)	Oral	1.4%	Cox *et al.* (1973)
typhimurium (10^6)	Oral	0/79	Cox *et al.* (1973)
		Albumen (Yolk)	
enteritidis 13a (10^9)	Oral	38/75 (23/75)	Gast & Beard (1990)
	Contact at 62w	10/35 (12/35)	Gast & Beard (1990)
	Oral	37/262 (25/262)	Gast & Beard (1990)
	Contact at 37w	8/63 (11/63)	Gast & Beard (1990)
	Oral	50/336 (52/336)	Gast & Beard (1990)
	Contact at 27w	10/64 (10/64)	Gast & Beard (1990)
enteritidis	Crop 20w	0/283	Humphrey *et al.* (1991)
	Crop 52w	1/223	Humphrey *et al.* (1991)
enteritidis 10^8	Oral	0/250 (Yolk)	Shivaprasad *et al.* (1990)
		Albumen (Yolk)	
10^6	Oral	3/314 (11/314)	Shivaprasad *et al.* (1990)
10^4	Oral	6/221 (0/221)	Shivaprasad *et al.* (1990)

Natural Infection		
enteritidis (ns)	11/1119 (<10/g)	Humphrey *et al.* (1989)
enteritidis (ns)	32/5790 (<20/ml)	Humphrey *et al.* (1991)

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* , The birds were inoculated either directly into the ovary, orally or by contact with other birds already infected with the organism.

w , The birds were inoculated at "w" weeks of age.

ns, dose not stated or unknown

the oviduct and the inclusion of blood spots in egg contents. Jeffrey and Pino (1943), however, noted no increase in the number or incidence of blood spots after exposing a laying flock to frequent disruption. Blood spots may, however, still be taken as evidence for the presence of contaminated material being included in the egg during formation. Those who have noted intermittent contamination of eggs with salmonella did not record a high incidence of blood spots in such eggs. "Calcium-splashed" eggs provides another example of an egg fault caused by disturbance. The chalky texture of the cuticle of affected eggs is considered to result from an egg being retained for an unusual length of time in the shell gland, probably as the result of physiological stress or violent disturbance in the laying house (Hughes and Gilbert 1984). As there is tentative evidence that stress and oviducal malfunction may well play a role in salmonella infection of eggs, this aspect ought to be considered in schemes intended to ameliorate the current problem of egg related salmonellosis in humans.

It is quite remarkable that the contemporary literature on *S.enteritidis* and hens' eggs rarely if ever makes reference to *S.hadar* and turkey eggs. In practice turkey flocks of one particular British firm were infected with *S.hadar*. Over several years there was a progressive increase in the number of humans who suffered from salmonellosis due to the consumption of turkey meat contaminated with this serotype. Suddenly and quite inexplicably the incidence of cases diminished to very

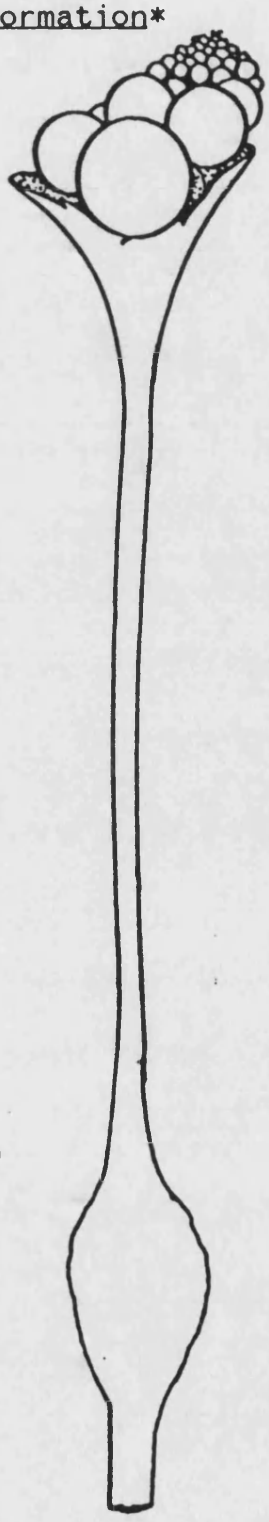
low levels. Vertical transmission of eggs was thought to be the cause of the perpetuation of *S.hadar* in this company's live stock. Baxter-Jones, who was the company's bacteriologist throughout this episode, stated recently (1991) that the culture of large numbers of eggs proved to be a "fruitless exercise" and that in his experience the egg transmission rate of this serotype was 1:10,000 to 1:100,000. This cautionary view ought to be considered by anyone who wishes to begin control policies based on the examination of eggs.

Oviducal infection of the egg

There are many difficulties involved in investigations of oviducal contamination. Rigorous precautions must be taken when handling the hens in order to ensure that the condition of the oviduct *post mortem* reflects its natural status (Haines 1939). The literature leaves the impression that suitable methods have not always been adopted to study this route of infection. For example the stress imposed on hens slaughtered in commercial processing plants may have exaggerated the infection rate with salmonella in the studies discussed on page 9. It is evident also that the flora of this organ cannot be considered independently from that of the eggs taken directly from the hen.

It is well documented that opportunities may well exist for an egg to become contaminated during passage (Figure 2) along the oviduct (Romanoff and Romanoff 1949). Rot producing bacteria were found in the oviduct

Figure 2. Egg Formation*



Infundibulum

8-9 cm
15-30 mins

Grasps ovule at ovulation Yolk acquires outer layer membrane, and chalazal layer of albumen.Probable site of fertilization, sperm maturation.

Magnum

30 cm

2-3 hours

Albumen secreting region

Isthmus

10 cm

60-75 mins

Synthesis of shell membranes, start of shell formation at junction of uterus (Mamillary cores)

Uterus
(shell gland)
4-5 cm

18-20 hours

Completion of shell, "plumping" occurs and pores formed. Shell pigments deposited.

Vagina

8 cm

Storage of sperm in "nests"

Oviposition

Egg expelled by prolapse of the posterior part of the uterus.

*adapted from Burley and Vadehera (1989)

by Lamson (1909) and, when such organisms were placed in oviducts they remained viable for 48-72 hours (Horowitz 1903). Studies of deliberately infected hens (inoculum 10^{8-9} salmonellas/ml) showed that a significantly greater number of contaminated eggs were laid by hens inoculated cloacally (9.15%) compared to those (3.78%) inoculated orally (Bale and Hinton 1991-unpublished). It may be inferred that cloacal contamination led to oviducal infection by one or other of the mechanisms discussed below.

In commerce the incidence of infection of the oviduct may be higher towards or at the end of lay (Harry 1963; Bruce and Drysdale 1991). The latter workers examined 656 fertile eggs collected over a 10-day period from 140 end-of-lay hens. The eggs which failed to hatch were examined microbiologically along with the oviducts of the producing hens at slaughter. It is apparent from Table 3 that 63% of oviducts were contaminated, but only 5.6% eggs produced therein were infected. Forty percent of the oviducts were contaminated at a level of $>10^4$ per organ. Of the eggs produced by the remainder of "uncontaminated" oviducts, 4% harboured organisms. Sesma *et al.* (1988) studied eggs from 2 layer flocks on small farms following 2 outbreaks of human salmonellosis. *Salmonella enteritidis* and *S.typhimurium* were isolated from the cloaca, liver, ovaries and oviduct of the hens. There was no correlation between the level of contamination of the oviduct and the incidence of contaminated eggs.

Table 3. The level of bacterial contamination found in the oviducts of hens at the end of lay and the incidence of contamination in eggs failing to hatch*

Bacterial count/ oviduct	No. birds	No. eggs laid	No. of eggs contaminated
nil	52	242	9
$<10^3$	19	100	0
$>10^3-10^4$	30	148	1
$>10^4-10^5$	7	31	0
$>10^5-10^6$	12	55	0
$>10^6$	20	80	4

* Drysdale (1985) from Bruce and Drysdale (1991).

There are several means whereby bacteria may gain entry to the oviduct. The inclusion of foreign bodies - such as feathers, faeces and stones - in eggs is taken as evidence that violent antiperistalsis is not an uncommon happening in this organ (Romanoff and Romanoff 1949). It is probably caused by contraction of the oviduct following the release of an egg. This may well force material including bacteria in the uterus upwards into the oviduct (Warren and Scott 1935).

Contraction of the muscles in the walls of the oviduct is essential for the transport of sperm from the vagina to the infundibulum (Van Demark 1953). Such contractions may be induced in response to a tactile stimulus. Non-motile or dead sperm as well as other fluids induced the same muscular contractions as did motile sperm. This stimulus is thought to operate only above the uterovaginal junction. Sperm deposited below this level were discharged through the cloaca (Allen and Grigg 1957). These contractions may account for anomolies such as double-yolk eggs (Asmundson 1931), eggs within eggs (Romanoff and Hutt 1945), as well as the transfer of bacteria from the cloaca to the oviduct (Haines 1939). Antiperistalsis may occur also at times of digestive disturbance (Romanoff and Romanoff 1949). Bacteria may also be transported up into the oviduct during major muscle movements caused by a stimulus other than those noted above. Thus, for example, one hour before oviposition some eggs are rotated through 180^{0} in a horizontal plane (Bradfield 1957). Van Drimmelen

(1951) states that this is responsible for 10-30% of eggs being laid blunt end first. The extent of such disturbances and their microbiological implications do not appear to have been discussed in the literature.

Bacterial contamination of the oviduct may occur at insemination (Haines 1939) due to infection of sperm deposited close to the cloaca (Lorenz 1966). Spermatozoa labelled with P^{32} and deposited in the uterus and vagina were found in the infundibulum one hour after insemination (Allen and Grigg 1957). Harry (1963) demonstrated that artificial insemination introduced *Escherichia coli* into the vagina. The organism was isolated subsequently from the albumen and yolk of eggs at oviposition. Generally more coliforms were found in the oviducts of artificially rather than naturally inseminated hens.

Sperm are suspended in seminal fluid derived from the blood plasma. It is of similar composition to lymph in terms of glucose, mineral and amino acid content (Lake 1966). Thus it would appear to be capable of sustaining the viability, but not necessarily the growth, of Gram negative bacteria. Indeed *S.enteritidis* have been shown to survive in a sperm suspension for 3 days at room temperature (Bolder and van Lith 1991). Sperm may remain viable in the hen for upto 15 days (Walton and Whetham 1933, Van Drimmelen 1945). Retention of viability is probably associated with the presence of "sperm nests" which nurture the functional capability and, according to some, maturation of sperms (Van Drimmelen 1951). Olsen

and Neher (1948) noted, however, that spermatozoa taken directly from the vas deferens of the male were capable of immediately fertilizing the ova. In other words the sperm did not require a period of maturation in the oviduct. Even so high numbers need to be present for fertilization to occur. It has been suggested that the "sperm nests" favour the accumulation of large numbers of sperm in the infundibulum and hence at the site of fertilization (Hammond 1940). Could such "nests" harbour salmonellas also ? There has been much speculation regarding the location of the "sperm nests". Lake (1969) suggested that spermatozoa are sustained in the epithelial gland of the utero-vaginal junction and that "packages" are released daily to ascend to the infundibulum where a succession of yolks are fertilized. Van Drimmelen (1946) has identified "sperm nests" in the infundibulum. He observed "nests" containing 50-80 sperm, arranged with their tails pointing out into the lumen. These were expelled as a result of the stretching of the infundibulum. It is well known that the infundibulum becomes active at the time of ovulation and fertilization occurs in this area following the release of the ovum from the ovary (Olsen and Neher 1948). The studies by Bolder and van Lith (1991) showed that sperm suspensions containing a heavy bacterial inoculum did not result in isolation of salmonella from either the eggs or the chicks produced therefrom.

The observations discussed above demonstrated that investigations of the outcome of oviducal contamination

of eggs pose practical problems. Obviously eggs cannot be infected with certainty in the oviduct when studying the fate of contaminants in their contents post oviposition. Consequently attempts have been made to adopt methods which simulate this route of egg infection. Such methods include the inoculation of organisms directly into the albumen of whole eggs (Kim *et al.* 1989), onto the air cell membrane (Brooks 1960) or yolk of whole eggs (Ayres and Taylor 1956, Humphrey 1991). The first of these methods simulates the situation in which albumen is contaminated on secretion in the magnum, and the second simulates the situation in which the shell membranes are contaminated during formation in the isthmus. The course of microbial infection following such methods of infection will be discussed subsequently (p30).

Control measures

Judging from the evidence discussed to date there is a need to ensure the minimal infection of feed and to detect and eliminate infected breeding flocks (Rodrigue *et al.* 1990). Indeed, the Ministry of Agriculture, Fisheries and Food emphasized the importance of possible transovarian infection and introduced the mandatory testing of poultry flocks in order to prevent the spread of *S.enteritidis* from breeding flocks *via* eggs to progeny. This involves the compulsory slaughter of infected birds as prescribed by the Zoonoses Order (Anon 1989). Regarding elimination of the organism from feed,

the introduction of the Disease of Animals (Protein Processing) Order (Anon 1981) has reduced the incidence of contaminated feed from 31% in 1982 to 14% in 1988 (Mason and Vines 1989). This order, however, does not include the vegetable protein, soya meal - the most common source of *Salmonella* in the study by Humphrey and Lanning (1988). The process of pelleting reduces the number of salmonella isolates from feed by 82% (Jones *et al.* 1991). The recommended heat treatment is 80 ^{0}C for 5 mins or 74 ^{0}C for 20 minutes (Anon 1989). The incidence may be reduced further by the addition of formic acid (0.5% w/w), propionic acid or other antimicrobial agents to feed. The addition of formic acid, for example, caused a reduction in the rate of isolation of salmonellas from 15.3% to 1.2% in hatchery waste (Humphrey and Lanning 1988). It would appear that the inclusion of low concentrations of an organic acid such as formic acid (0.25-0.75% of 85% formic acid) is capable of eliminating *Salmonella* carriage in chicks (Hinton, Linton & Perry 1985), and may limit the horizontal spread of *S.enteritidis* (Humphrey *et al.* 1991).

Nurmi and Rantala (1973) suggested that colonization of the hen's intestine could be avoided by orally administering at hatching cultures of anaerobic bacteria derived from the faeces of mature birds. Faecal cultures have been found to provide protection 30 min to one hour after administration. Maximum protection was conferred on the chick 6-8 h after administration (Stavric *et al.* 1987). It provided protection against infective doses of

10^3-10^6 salmonellas (Pivnick and Nurmi 1982). In Finland competitive exclusion has been widely adopted. The use of products such as "Broilact", a mixture of anaerobic bacteria isolated from the faeces of mature birds are fed to newly hatched chicks. Indeed Finnish law states that every broiler flock must be examined serologically for the presence of salmonellas. Since the integrated use of products such as "Broilact" began in August 1991, no salmonellas has been found on farms that used it (Schneitz 1992). It needs to be stressed, however, that Finland is a small country and the success of the Nurmi concept may be linked to the small scale of its poultry industry. It is generally considered that application of the competitive exclusion principle - competition for binding sites on the epithelial cells of the gastrointestinal system - would only be successful if used as part of a larger programme to control salmonellas (Mead and Impey 1984).

In the short term antibiotic therapy may be needed to eliminate depots of salmonellas in breeding flocks. In this instance, of course, care must be taken to minimize the risk of selecting plasmid-borne resistance and transferring organisms with this attribute along the production chain to the consumer. Antibiotic resistance in salmonellas is associated with plasmids acquired as the result of a selective pressure caused by the use of a specific antibiotic (Threlfall and Frost 1990). So far, however, resistance to antimicrobial agents is rare in *S.enteritidis* of poultry origin. In 1987 only 10.7% of

isolates in England and Wales were resistant to multiple drugs (Frost, Ward and Rowe 1989). It has been surmized that *S.enteritidis* carries a specific restriction system which destroys incoming DNA (Anderson and Threlfall 1970). Plasmids that are phage-type determining, however, may convert strains of PT 4 to PT 24. In 1988 97% of PT 24 strains were resistant to tetracycline.

In the US the failure of the Voluntary Model State Program, introduced by the USDA/Food and Drug Administration, has led the USA Department of Agriculture to ammend its regulations to include *S.enteritidis* as a cause of communicable disease of poultry. This involves restricting movement of flocks found positive - based on the recovery of the organism from internal organs - and the updating of the National Poultry Improvement Plan (NPIP), initially set up in 1935 to deal with the problem of *S.pullorum*. This states that all egg producing flocks must be classified as "US Sanitation Monitored". Approximately 90% of the breeding and hatchery industry participate in serological testing of fowls and the classification of various disease control programmes which, up until the addition of *S.enteritidis*, included *S.pullorum, S.gallinarum, Myocoplasma gallisepticum, M.synoviae and M.meleagridis.*

The 1990 conference of the NPIP (Anon 1991a) approved changes (not yet published) in the Code of Federal Regulations which included bacteriological testing of the environment (layers) every 30 days using improved culturing techniques with the aim of eliminating

S.enteritidis from layer breeding flocks. It was suggested that this could be achieved by the use of *Salmonella*-free feed, ie. pelletized feed should contain no animal protein unless it was treated under the "Animal Protein Products Industry *Salmonella* Education/Reduction Programme". This involves the exposure of feed to temperatures of 87.7 ^{0}C or above, or 84.8 ^{0}C with 70 lb of pressure or 73.8 ^{0}C for 20 minutes. In the case of mash feed, the animal protein must be treated as for pelleted feed and then crumbled into the mix. Bacteriological samples should be taken from the birds at 4 months of age and every 30 days thereafter. If *S.enteritidis* is found in the environment or demonstrated serologically in the birds then 60 live birds are examined. If the flock is found positive then certification of the flock by the NPIP is suspended, until the flock is found to be *Salmonella* free.

In general positive flocks are destroyed or their eggs diverted to pasteurizing plants. (This latter policy is now being considered in the UK). The FDA has also published codes for retail food establishments which redesignated eggs as a potentially hazardous food-stuff, and recommended that they be refrigerated at all times. It also cautioned against the use of pooled eggs, emphasizing temperature regulation of the food-stuff before and after cooking (Anon 1991a).

Antimicrobial defence of the egg

The redesignation of an egg as a potentially hazardous foodstuff that needs refrigerated storage implies that its contents may be easily colonized by large numbers of salmonellas. The evidence for and against this contention is reviewed below.

As noted above, an egg may become contaminated with salmonellas by any one of 3 routes, transovarian, oviducal or trans-shell (Duguid and North 1991). With the last 2, the organisms have to survive the antimicrobial properties of the albumen if they are to pose a threat to a consumer. This section reviews work done on the antimicrobial defence of the hen's egg. Haines (1939) noted that "when the avian egg is laid it is a complete structure equipped for the dual purpose of nourishing the developing embryo and defending it against microbial invasion". Over the years speculation has linked many components of the hen's egg (Table 4) with this defence system. In practice studies of the defence of the yolk - the main depot of the material required for embryogenesis - has led to the recognition of 3 major components of the defence and 3 stages in its negation and the development of gross contamination of an egg's contents. 1. Contamination and penetration of the shell. 2. Colonization of the shell membranes. 3. Gross infection of the albumen and yolk without necessarily any visible change in either (Board and Fuller 1974). It has been suggested that infection/rotting of shell eggs is the result of mishandling by man leading to an

Table 4. Contribution of the components of the hen's egg to the antimicrobial defence of its contents and the developing embryo*

Component	Contribution to defence	
	Physical	Antimicrobial
Cuticle, shell & shell membranes	Protection of embryo against crushing Prevention of water logging Camouflage	Barrier to microbial invasion
Air cell	Compensation for changes in pressure and volume of an egg's contents	
Thin white & Albuminous sac	Protection of embryo against physical damage Lag against changes in ambient temperature	Holds yolk in a central position Viscosity an impediment to bacterial movement Control of the rate and extent of microbial growth Passive immunity of chick
Chalazae & Chaliferous membrane		Holds yolk in a central position
Vitelline Membrane		Physical isolation of yolk from white
Yolk	Location of young embryo at least distance from heat	Passive immunity of chick

* Adapted from Board & Fuller (1974).

overloading of the natural defence system (Brooks and Taylor 1955, Mayes and Takebali 1983).

The following discussion of the antimicrobial properties of the egg covers the course of infection of those contaminated *via* the trans-shell route where organisms are confronted by the entire antimicrobial defence system (Tables 4 and 5). The incidence of salmonellas on an egg shell or in its contents will be determined by 3 factors: (1) the environment into which an egg is laid; (2) the structure of the shell, and (3) the handling and storage of an egg (Rizk, Ayres and Kraft 1966).

Physical Defence

The structure of the shell (Figure 3) is the major determinant of the incidence of contamination of an egg's contents by the trans-shell route. The shell of the hen's egg is composed mainly of calcium carbonate (98% by weight) - in the form of calcite - together with traces of magnesium, phosphate, an organic matrix and water (Board and Sparks 1991). The inner (cone) layer consists of conical projections which merge with the outer shell membrane. The major part of the shell (the palisade layer) is formed from polycrystalline columns of calcite. Pores of a straight posthorn morphology run radially across this layer. The shell's surface and the outer orifice of the majority of the 7,000 -17,000 pores are covered completely or partially by the cuticle. This is a thin layer of glycoprotein consisting of spheres when

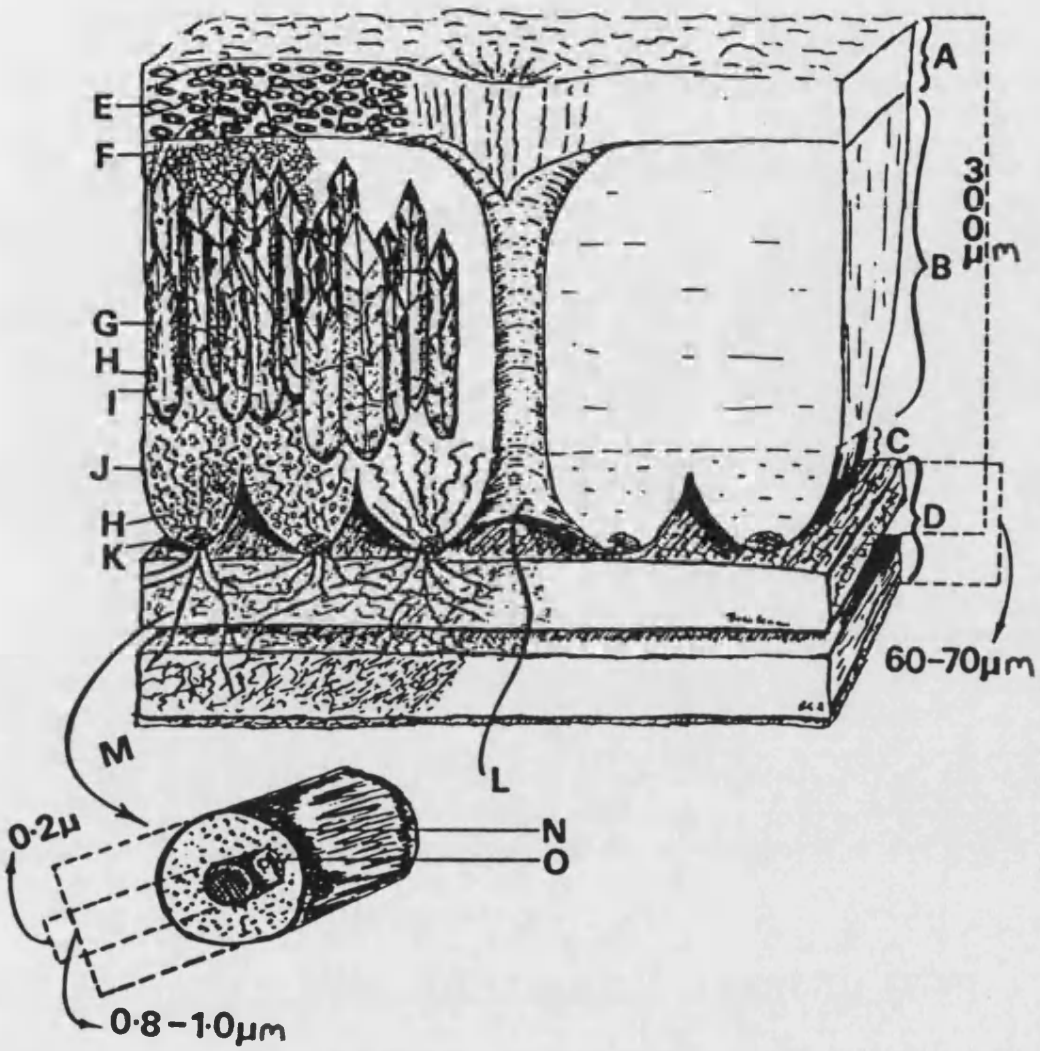


Figure 3.

An artists impression of the organization of the egg shell as shown by radial section through a pore.
A, Cuticle; B, Spongy layer; C, Mammillary layer; D, Shell membranes; E, vesicles and fibres; F, Amorphous crystalline materials; G, Columns of calcite crystal; H, Fibres; I, Vesicles; J, Amorphous crystalline material; K, Protein plug; L, Pore canal; M, Large fibres; N, Mantle; O, Keratin core.

Board (1969)

in the mature state (Tyler and Geake 1953, Board and Halls 1973, Nascimento 1991).

Organisms may become trapped in the immature cuticle as an egg passes through the cloaca (Stuart and McNally 1943). If a bird is actively transmitting pathogens such as salmonellas in its faeces, then the shell may well harbour these pathogens also (Jones *et al.* 1991). Bacteria have been shown to penetrate the shell readily when the cuticle is still wet and hence immature, a state of very short (1-3 min) duration (Sparks and Board 1985). Organisms trapped in the cuticle may penetrate the shell as the former deteriorates during storage (Vadehera, Baker and Naylor 1970).

There is ample evidence that the egg shell enveloped in a mature cuticle impedes the inward movement of water and of micro-organisms located on its surface (Williams and Whittemore 1967). The cleanliness of the environment into which an egg is laid is of course an important determinant of the extent of microbial contamination and hence penetration of the shell. Up to 15 times more bacteria have been found on eggs from deep litter as opposed to those from battery cages (Harry 1963). Jones *et al.* (1991) observed that the incidence of shell contamination could be diminished by reducing the moisture content of nest litter. Moreover the incidence of contamination of the contents of eggs laid on the poultry-house floor is greater than that of those laid in nest boxes (Smeltzer *et al.* 1979, Padron 1990, Bruce and Drysdale 1991).

Drysdale (1985) examined shell thickness, porosity - measured as water vapour conductance, and cuticle deposition on eggs produced by a broiler-breeder flock. Eggs were examined during the hens' 29^{th}, 35^{th} and 50^{th} weeks of age. The results did not demonstrate any relationship between contamination of the egg contents and thickness of the shell. These results are in accord with those of Vadhera *et al.* (1970) and Smeltzer *et al.* (1979). Moreover there was no evidence that a high level of porosity caused a higher incidence of contamination of the contents. These results are in agreement with those of Sparks and Board (1984). The cuticle enveloping the outer surface of the shell was the main impediment to bacterial penetration of the shell, an observation in agreement with those of many earlier workers (eg. Romanoff and Romanoff 1949, Fromm 1967, Sparks and Board 1985). Drysdale (1985) noted that 40% of eggs that failed to hatch had a poor cuticle and a high incidence of contamination of their contents.

Even if an egg has an intact cuticle there are 10-20 pores, out of a total of 7,000 to 17,000 (Tyler 1953) which are prone to flooding and hence bacterial infection of the contents (Orel 1959). These pores are probably of prime importance, particularly when eggs are washed, in allowing the passage of micro-organisms from the shell surface to the underlying shell membranes (Board and Board 1967). Paton and Ayres (1964) used "Calcofluor-White M2R" to demonstrate the passage of *Salmonella* across the shell and into the membranes. The fluorescent

brightener was taken up by growing organisms without apparent inhibition or mutagenesis. Salmonellas that penetrated the shell fluoresced and those restrained by the shell membranes formed colonies that fluoresced in UV light.

Organisms in faeces appear to penetrate the shell and gain access to the underlying membranes more easily than those in water alone (Bruce and Drysdale 1991). Faeces may increase the extent of microbial penetration of the shell and cuticle or enhance the growth of contaminants on the shell membranes by supplying these with Fe^{3+} and other growth factors (Bruce and Drysdale 1991, Clay and Board 1992). Padron (1990) exposed eggs to sterile faeces which had been sprayed with *S.typhimurium* and he increased the incidence of contamination of the chorioallantoic membranes and yolk sacs of incubated eggs. Similar results were obtained following contact of eggs with deliberately contaminated nest litter for 10 minutes.

Infection of an egg's contents may be the result of egg washing. Bacterial translocation across the shell is probable if the wash water is colder than the egg (Haines and Moran 1940, Büchli 1967, Moats 1978). The temperature differential causes a slight negative pressure because the reduction in the volume of the contents is greater than that of the shell (Haines and Moran 1940). This results in a small amount of contaminated water being drawn into the egg (Sparks and Board 1984). When using this technique, Stokes *et al.*

(1956) demonstrated that *S.oranienberg, S.typhimurium, S.montevideo, S.pullorum* and *S.gallinarum* penetrated the shell and, with storage at 29 ^{0}C, large populations developed in the eggs contents. They contended that initially salmonellas were sucked into but not through the shell membranes, such that the contaminating organisms were separated initially from the antimicrobial factors in the albumen. Eggs contents do not always become contaminated, however, when washed in *Salmonella*-infected water (D'Aoust, Stotland and Randall 1980). The reasons for this are not known.

If egg washing is done correctly - ie. the temperature of the wash water (containing very low levels of iron) is 20 ^{0}C higher than that of the eggs and the eggs are dried as quickly as possible (Büchli 1967), then the rate of infection will be akin to that of unwashed eggs (Moats 1979, Forsythe, Ayres and Radlo 1953, Ostlund 1971). Washing, however, may render the egg more susceptible to subsequent bacterial infection, especially if the cuticle has been damaged by the washing process and eggs are stored under moist conditions (Trussell, Triggs and Greer 1955).

Once organisms penetrate the cuticle and shell, the shell membranes act as an additional mechanical barrier to contamination of an egg's contents (Stokes and Osborne 1956). An egg shell freed of membranes does not restrain the passage of bacteria as effectively as an intact one (Garibaldi and Stokes 1958).

The membranes are composed of anastomising fibres having an elastin-like core and glycoprotein mantle (Leach, Rucker and Van Dyke 1981). Tranter and Board (1982) noted the adhesion of bacteria to the glycoprotein mantles of the membrane fibres. This adhesion may be one reason for the transient impediment of microbial movement across the membranes and into the underlying albumen. This property is lost 18-24 h after contamination (Walden, Allen and Trussell 1956). The reasons for this change are not known and speculation that bacterial proteases may be involved have not been supported by the observations of Garibaldi and Stokes (1958) and Board (1965). The membranes probably contain nutrients that support the growth of organisms (Stokes and Osborne 1956). Bacterial multiplication has been observed on egg shell membranes *in vitro*. Board (1965), for example, demonstrated that intact shell membranes in buffered mineral salts supported the growth of rot-producing bacteria and that *Aeromonas liquefaciens* digested this structure.

Two factors may play an important role in bacterial penetration of the egg contents. (1) The nature of substances deposited on the membranes along with the contaminating organism. For example, contamination of membranes *in situ* with faecal extract (Clay and Board 1992). (2) The protection against the antimicrobial properties of the albumen afforded the organism by the network structure of the membranes (Stokes *et al.* 1956). Both of these are discussed below.

Thus in summary, contamination of the eggs contents occurs when the cuticle, shell and shell membranes fail to prevent microbial penetration (Mayes and Takebali 1980).

Antimicrobial properties of the albumen

Bacteria which penetrate the physical defences discussed above are confronted by a range of albumen proteins (Table 5) having antimicrobial properties (khoury-Doughly, Spencer and Verstrate 1976). Fresh egg white added to a nutrient medium, for example, inhibited the growth of *S.pullorum,* the extent of inhibition being directly proportional to the amount of added white (Lutsky and Bell 1953).

Although the antimicrobial properties of many of the proteins isolated from egg albumen have been demonstrated, there is little doubt that the unavailability of iron through chelation by ovotransferrin is the cardinal impediment to bacterial growth in albumen (Brooks 1960, Board 1964, Tranter and Board 1982). Additionally, the very small amounts of non-protein combined nitrogen (Haines 1939, Brooks 1960, Board and Halls 1973) in the fresh egg is at or below the limiting threshold for bacterial growth. The alkaline pH of the white also contributes to the antimicrobial nature of the albumen (Banwart and Ayres 1957). With diffusion of CO_2 from the albumen through the pores in the shell, the pH of the hen's egg albumen changes from pH 7.6 to 9.4 in the week following oviposition. The rate of

Table 5. The antimicrobial properties of the albumen proteins

Protein	% of total protein	Biological property	Reference
Ovotransferrin	13	Chelates di & tri-valent ions Fe,Cu,Mn etc.	Osborne & Campbell (1900) Schade & Caroline (1944)
Ovomucoid	11	Inhibition of trypsin	Neumeister (1890) Balls & Swenson (1934) Lineweaver & Murray (1947)
Lysozyme	3.5	Hydrolyses β1-4 linkage in peptidoglycan. Complex with Ovomucin	Laschenko (1909) Flemming (1922) Alderton *et al.* (1945)
Ovomucin	1.5	Anti-viral heamagglutination. Complex with Lysozyme	Eichhloz (1898) Osborne & Campbell (1900) Brooks & Hale (1959)
Ovoinhibitor	0.1	Inhibits proteases	Matsushima (1958)
Ficin-Papain inhibitor (Cystatin)	0.1	Inhibits proteases	Fossum & Whitaker (1968) Barrett (1981)
Avidin	0.1	Chelation of biotin	Boas (1924) Eakin, Snell & Williams (1940)
Thiamin-binding protein	0.1	Chelation of thiamin	Muniyappa & Adiga (1979)

change is determined largely by the temperature at which an egg is stored (Sharp and Whitaker 1927). Most common contaminants of eggs were killed in albumen adjusted from pH 6-8 to pH 9-10 (Banwart and Ayres 1957) - the optimum pH for the growth of *Salmonella* spp. is 6.5 to 7.5 (Chung and Goepfer 1970). The alkaline pH of the albumen accentuates also the antimicrobial action of ovotransferrin (Schade and Caroline 1944).

The stucture of the albumen, especially the albuminous sac, contributes in 2 ways to the antimicrobial defence of an egg. It ensures that the yolk in an egg at oviposition is located centrally and it impedes bacterial movement (Cotterill and Winter 1954, Brooks and Hale 1959, Tranter and Board 1982). Both of these attributes stem from the viscosity of the albumen which is due to an interaction between lysozyme and ovomucin that is accentuated by alkaline conditions (Robinson and Monsey 1972, Tranter and Board 1982). Ovomucin consists of 2 types of glycoprotein (Robinson and Monsey 1975). The F component (carbohydrate rich) interacts with lysozyme to a greater extent than the S component (carbohydrate poor) by electrostatic attraction between the negative charges of the terminal sialic acid and the positive charges of the lysl Σ-amino groups of lysozyme (Kato, Imoto and Yagashita 1975).

It has been suggested that in eggs stored at temperatures >10 ^{0}C, the growth of organisms in eggs occurs only when the yolk makes contact with the infected shell membranes (Board 1964). This occurs as the result

of the breakdown of the albuminous sac. The F component of ovomucin is released with time and the gel structure of the albuminous sac contracts and the yolk floats towards the shell membranes (Kato and Sato 1972, Tranter and Board 1982). The rate of upwards movement of a yolk in eggs is determined by the rate of loss of the viscosity of the albuminous sac (Zagavsky and Lutikova 1944, Ayres and Taylor 1956). Vadhera *et al.* (1970) demonstrated that it was the location of the nidus of infection relative to the direction of movement of the yolk - ie. towards the air cell in eggs stored broad pole uppermost, which determined the rate of onset of gross infection of the albumen. Indeed one may conclude from the available evidence that it is the rate of decay of the albuminous sac that underlies a very common observation, namely that there is a 10-20 day lag between shell penetration and macroscopic changes in the egg contents (Table 6). This lag period is common to rot-producing bacteria (Gillespie and Scott 1950) and salmonellas (Bigland and Papas 1953, Stokes *et al.* 1956).

Ovotransferrin

Initial contaminants of the albumen are considered to remain quiescent for the lag period noted above. Quiescence is due almost entirely to the properties of ovotransferrin (originally termed conalbumin by Osborne and Campbell 1900). The antimicrobial attribute of ovotransferrin was first recognised by Schade and Caroline (1944). They noted growth inhibition of

Table 6. Time taken from the penetration of the shells of fresh eggs and the occurrence of gross contamination or macroscopic changes in the albumen

Test Organisms	Infection Method	Temp (^{0}C) (Days^{+})	Reference
Bacillus prodigious	Immersion*	15-18	Zagaesky &
pyocyaneus	"	(15-20)	Lutikova(1944)
Pseudomonas	Immersion	20 (14)	Gillespie & Scott (1950)
Salmonella bareilly	Rolled in	22	Bigland &
oranienberg	faeces	(6-16)	Papas (1953)
kentucky	containing		
typhimurium	test organism		
rot producers	natural & artificial (not specified)	7-37 (14)	Miller & Crawford(1953)
Ps.ovalis	Immersion	15 (7-11)	Elliot (1954)
Salmonella oranienberg	Immersion	29	Stokes,Osborne
typhimurium		(15)	& Bayne (1956)
montevideo			
pullorum			
gallinarum			
Pseudomonas	Immersion	20 (21)	Orel (1959)
Ps.aeruginosa	Immersion	26 (10-15)	Fromm & Monroe (1960)
Psuedomonas ovalis	Immersion	15	Garibaldi &
fluorescens		(25)	Bayne (1960)
Pseudomonas	Air cell£	20 (13)	Brooks (1960)
Ps.fluorescens	Air cell	27 (12-30)	Board (1964)
Seratia marcesens	Air cell	30 (10-15)	Board & Ayres (1965)
S.lexington	Immersion	22-25	Rizk, Ayres &
S.anatum		(14)	Kraft (1966)
Ps.aeruginosa	Immersion	21-23 (15)	Vadehera *et al.* (1970)

S.enteritidis	Air cell	25 (12-34)	Clay & Board (1991)
S.enteritidis	Air cell	20 (15)	Dolman & Board (1992)

+, The number of days before the occurence of gross contamination or macroscopic changes in the albumen

*, The eggs were immersed in a suspension of bacteria

£, The air cell was located by candling, a hole drilled in this area and the air cell membrane inoculated with the test organism

bacteria and yeast in nutrient broth supplemented with egg albumen at pH 7.4 or higher. Iron alone overcame this inhibition.

Ovotransferrin belongs to a group of closely related iron-binding glycoproteins (transferrins) found in milk, other mammalian secretions, serum and avian egg white. A common feature of these proteins is their ability to bind 2 ferric iron atoms per molecule (Aisen, Leibman and Reich 1966) at 2 metal-binding sites (Aasa *et al.* 1963) situated at the N and C terminal halves (Williams 1974, Keung *et al.* 1982). It is likely that the C terminal site has a higher affinity for iron, its binding ability being determined by pH (Aisen, Liebman and Zweir 1978). Schade, Reinhart and Levy (1949) demonstrated that CO_2 was involved in the formation of iron complexes of human serum transferrin. It was believed that CO_2 activated the metal binding sites (Aisen *et al.* 1966). Therefore, for each metal ion bound to ovotransferrin *in vivo* a bicarbonate or carbonate ion binds simultaneously, probably between the metal and the protein. This stabilizes the bond and protects it from hydrolysis (Williams and Woodworth 1973). It is probably responsible also for the formation of the salmon-pink colour complex (Warner and Weber 1953, Aisen *et al.* 1966), having maximum absorption at 400-470 nm (Schade *et al.* 1949). The transferrins form complexes with other transition metals, for example, zinc (Warner and Weber 1953). All complexes dissociate in acid - <pH 6.5 -

(Tengerdy, Azari and Tengerdy 1966), but not in alkaline conditions - pH 9-10 - (Tan and Woodworth 1969).

Ovotransferrin is proposed to exist in 3 forms, iron-free, complexed with one iron ion and complexed with 2 iron ions (Aisen *et al.* 1970). The last mentioned is the more stable state with regard to organic solvents, high pressure and denaturation by heat (Azari and Feeney 1961).

The unavailability of iron through chelation by ovotransferrin is the main impediment to the growth of bacteria in albumen (Schade and Caroline 1944, Brooks 1960, Garibaldi 1960). Indeed Board, Hendon and Board (1968) noted that the addition of iron to an infected egg shell membrane promoted extensive bacterial multiplication and heavy infection of the albumen. It has been observed also that where naturally or artificially iron-contaminated wash water was used, gross infection of the egg contents occurred rapidly (Garibaldi and Bayne 1960).

Neilands (1974) states that "Life of any form without iron is in all likelihood impossible". Iron has been found to be involved in practically every aspect of growth and metabolism. It's real importance in microbial life is its role as a co-factor in enzyme reactions. For example iron acts as a catalyst in the aerobic metabolism of common carbon sources and in the synthesis of growth enzymes (Coughlan 1971). Gram negative bacteria require from 0.02 - 0.1 µg/Fe/ml (0.36 -1.8 µm) to maintain growth. Thus deficiency of iron generally results in

partial or complete inhibition of growth (Rowe-Byers and Arceneaux 1976). The iron content of eggs ranges from 1.76 to 2.44 mg/100g, the mean iron content of albumen being 0.0028 mg/100g (Johnston 1956).

Due to the extreme insolubility of ferric iron at alkaline pH's, however, aerobic and facultative organisms may be forced to synthesize affinity systems for the acquisition of iron from the environment. At free iron concentrations of below 1 µg, either hydroxymates or phenolates are synthesized - Neilands (1974) proposed the general term, siderophores. Garibaldi (1970) speculated that organisms would excrete iron-binding compounds to negate the properties of ovotransferrin in eggs. He surmized that these iron transport compounds diffused into the albumen and scavenged iron thereby facilitating bacterial multiplication. For example pseudomonads production of fluorescence in albumen (Elliot 1954) has been considered to be associated with the scavenging of iron (Garibaldi 1970). Although such claims have been made, Tranter and Board (1982) were unable to demonstrate the presence of iron-binding compounds in albumen.

Salmonella spp. synthesize iron sequestering agents of the types noted above. These are excreted under low iron concentrations (Pollack and Neilands 1970) and their production is regulated by the amount of cell-associated iron (McIntosh and Earhart 1977). The phenolate siderophore, enterochelin (O'Brien and Gibson 1970), is a cyclic triester consisting of 3 residues of 2-3 dihydroxy-N-benzoylserine (Rowe-Byers and Arceneaux

1976). If *Salmonella* spp. are grown in an iron deficient medium, a rapid intracellular accumulation of the element occurs in the first 2 hours of growth. Little uptake occurs during the exponential phase. Thus the cellular iron content gradually decreases with each generation. When the level falls below 400 ng/mg (dry weight) of cells, the simultaneous onset of the synthesis of 3 outer membrane polypeptides and enterochelin occurs (McIntosh and Earhart 1977).

The uptake of iron by salmonellas requires the synthesis of several specific proteins by the organism. These include the enzymes required for the synthesis of enterochelin itself (Young, Cox and Gibson 1967), the outer membrane proteins required for binding the iron-enterochelin complex (Ichihara and Muzushima 1977), the proteins required for the translocation of this complex to the cytoplasm (Cox *et al.* 1970) and the cytoplasmic proteins required to release iron from the complex (O'Brien, Cox and Gibson 1971). Once transported the chelate probably remains within the cell pool of available iron, being reduced or released by enzymatic hydrolysis of the chelating agent (Rowe-Byers and Arceneaux 1976). Garibaldi (1970) noted that the addition of iron transport compounds to albumen permittted salmonellas to eventually reach dense populations at growth promoting temperatures (Weinberg 1979). The temperature probably determines the amount of iron required for growth and/or survival of the organism (Perry and Weinberg 1973). For example, *S.typhimurium*

has a reduced rate of enterochelin synthesis at 42 ^{0}C, leading to an impairment in the organism's ability to grow (Worsham and Konisky 1984). Infact, many Gram negative bacteria have reduced growth rates at elevated temperatures unless the growth medium is supplemented with iron (Garibaldi 1972). It has been concluded that growth at elevated temperatures affects the expression of the genes which determine the rate of enterochelin-iron transport in cells under iron stress (Worsham and Konisky 1984). Also, the lower the metabolic growth rate - eg. at reduced temperatures (8 ^{0}C) - the greater the decrease in enterochelin production (Perry and Weinberg 1973). This effect of temperature may have a biological role in enhancing the antimicrobial defence of eggs at or near the bird's body temperature of 42 ^{0}C

The pathogenicity of an organism *in vivo* may depend upon its ability to obtain iron held by transferrin. Infact, an adequate production of iron chelators by the organism may be essential for virulence (Miles and Khimji 1975). The excretion of iron binding compounds may be related directly to their degree of virulence (Bullen, Rogers and Griffiths 1978).

Although some workers (eg. Tranter and Board 1982) have considered that siderophores play no role in the infection of egg contents, observations made at the end of this study (p135) suggest that this topic needs reinvestigation.

The hen, immunity and *Salmonella* spp.

Antibody transfer occurs from the hen to the egg (Rose, Orlans and Buttress 1974). In 1901 Dzierjgowski found that, after injecting hens with diptheria antitoxins, antibodies passed into the yolks of their eggs. These were found to be concentrated in the water soluble fraction of the yolk. Hens have also produced eggs containing antibodies to bovine serum albumin (BSA) 2 days after being injected with BSA (Patterson *et al.* 1962). Transfer continued for up to 280 days post immunization. In other words, the transfer of antibody from hen to eggs may continue throughout a complete laying season (Buxton 1952). Rose *et al.* (1974) stated that minute amounts of antibody occur in egg white also.

Chicken serum contains 3 classes of immunoglobulins IgM, IgG (Leslie and Clem 1969) and IgA (Lebacq-Verheyden, Vaerman and Heremans 1972). IgA is found in chicken serum at a concentration of 0.33 mg/ml, less than 4% of the total immunoglobulin present compared to IgM at 2.55 mg/ml. IgA is the major immunoglobulin component in chicken bile and intestinal secretions. It is not found in saliva, tears or seminal fluid (Lebacq-Verheyden *et al.* 1972, 1974). As most *Salmonella* serotypes are confined mainly to the alimentary tract, it is considered that IgA plays an important role in their clearance from this site (Barrow 1991).

Serum *gamma* globulin is transferred by the folicular epithelium of the ovary to the developing ova (Patterson *et al.* 1962). Transfer probably occurs during the 4-5

days preceding ovulation, ie. during the most rapid growth phase of ovules (Patterson *et al.* 1962). The concentration of antibodies in the yolk is much less than that in the serum albumin (Buxton 1952).

Elisa systems have been developed to detect IgG antibodies to *S.enteritidis* and *S.typhimurium* in the yolk of hens' eggs (Nicholas and Andrews 1991, Dadrast, Hesketh and Taylor 1990). A positive correlation between infection with salmonellas and a high Elisa reading has been found. McLeod and Barrow (1991), for example, demonstrated that only 30 eggs were required to identify an infected flock in which the birds showed no evidence of infection. It would appear from other studies, however, that birds carrying antibodies from previous salmonella infections have undetectable levels in the yolk (Nicholas and Andrews 1991). Dadrast, Hesketh and Taylor (1990) suggest that *S.enteritidis* may grow in the yolk despite high levels of circulatory antibodies in the sera of the hen and hence the yolk.

It is also possible that anti-siderophore receptor antibodies exist. These block siderophore-mediated iron uptake and thereby inhibit bacterial multiplication under iron-restricted conditions (Griffths *et al.* 1985). The antibody involved is not considered to be secretory IgA, as it has not been shown to enhance the growth-inhibitory effects of lactoferrin (Samson, Mirtele and McClelland 1979). Normal human serum does contain, however, enterochelin-specifc immunoglobulin - for example serum inhibition of *S.typhimurium* was alleviated by the

addition of iron-free enterochelin (Yancey, Breeding and Lankford 1979).

In fertilized eggs from vaccinated hens, antibodies pass from the yolk to the embryonic serum from the 11^{th} day of embryogenesis onwards. These antibodies are considered to be absorbed from the yolk through the vitelline membrane and hepatic portal circulation and not *via* the yolk stalk or intestine (Buxton 1952). Antibodies may diffuse outward through the weakened vitelline membrane into the albumen as the unfertilized egg ages (Kramer and Cho 1970).

Birds which had been exposed to *Fusarium* mycotoxin (T_2) had significantly reduced resistance to colonization with salmonellas (Jones *et al.* 1991). The number of *Salmonella*-positive birds diminished as the birds aged (Morris *et al.* 1969). This was attributed to antigen specific IgA preventing the adhesion of bacteria or viruses to receptors on the surface of intestinal epithelial cells. Hens previously exposed to *Fusarium* maintained an immune response and transferred it passively to their chicks by secretion into the egg contents in the oviduct (Schat and Myers 1989).

The contributions of IgM and IgA in the defence system of the hen are largely unknown. Four-day-old chicks challenged with *S.typhimurium* F98 did not manifest signs of disease. Even so the organism was excreted for several weeks. Titres of serum IgG, IgM and IgA rose within a few days of infection and peaked at 4 weeks. The IgM titre rose quicker than those of IgA or IgG. The

concentration of IgM and IgA decreased after a few weeks but the IgG titre persisted. Titres of IgA were higher than those of IgM or IgG in intestinal washings and bile. There was no evidence of IgG in bile (Hassan 1991).

Turkeys vaccinated with inactivated *S.hadar* produced high levels of IgG antibody. It was passed to the egg and ultimately to the developing poults during incubation. Protection against infection of the hatched poults was greater than that exhibited by poults hatched from the eggs of unvaccinated hens (Thain, Wilding and Cullen 1984). Turkey chicks challenged with inactivated *S.typhimurium* ($1 \times 10^4 - 8 \times 10^7$/bird) at maturity had a marked resistance to subsequent infection and no morbidity was recorded (Truscott 1981).

Kawakiama, Osawa and Mitsuhashi (1966) attempted to isolate the factors in live vaccine which protected the host from infection by a virulent strain of *S.enteritidis*. They surmised that live bacteria may contain immunizing substance(s), possibly a heat labile protein (Toxin L), which is lost in vaccine production. The efficacy attained using the toxin-L for immunization, however, enhanced protection but not to the level of that of a live vaccine. They deduced that some other factor must be involved. Mice administered live vaccines intravenously or intragastrically developed an anti-salmonella immunity greater than those given attenuated vaccines *via* the same routes (Collins and Carter 1972). It became evident also that full immunity was only induced if the lipopolysaccharide of the mouse typhoid

cell had complete O-antigen side chains (Germanier 1972). Chart *et al.* (1990) confirmed this observation in chicks. They observed that birds infected naturally with *S.enteritidis* PT 4 had antibodies of the IgG class bound to *S.enteritidis* lipopolysaccharide antigen O (12). These birds produced eggs contaminated with *S.enteritidis.*

In general live attenuated vaccines are considered to produce better immunity than dead ones. Mitsushai *et al.* (1966) achieved a strong immunity by immunizing mice with live, highly virulent *S.enteritidis.* The cells in the liver, subcutaneous tissue and peritoneal cavity exhibited immune activity. Indeed they were capable of suppressing intracellular proliferaton and digested the organism in the absence of immune sera. This was deemed to be a form of cellular immunity, as in collaboration with complement and lysozyme, it suppressed the growth of *S.enteritidis in vitro.* Benjamin *et al.* (1991) used a *Salmonella* serotype isolated from an egg-associated outbreak of human salmonellosis (St Louis *et al.* 1988) to immunize (2 doses of 3×10^6 and 6×10^7/chick) one-week-old leghorn chicks. *Salmonella* was cleared rapidly and the number of organisms in the liver and spleen was <0.0001% of the initial inoculum after 17 days. The extent of protection appeared to be related directly to the size of the initial immunization dose. They concluded that an ideal vaccine would be a highly virulent, attenuated invasive mutant of *S.enteritidis.*

Immunization against invasive colonization is one means of preventing the spread of *S.enteritidis* from poultry to eggs (Benjamin *et al.* 1991, Nagaraja *et al.* 1991). The initial control of salmonellosis in hens probably lies in an improved understanding of the gut associated lymphoid tissue (GALT). Indeed the infection of hens with *S.enteritidis* may not be controlled immunologically until factors contributing to intestinal immunity are understood. It is generally agreed, however, that the best immunogen against infection with invasive salmonellas is a live vaccine that elicits protective cellular immunity (Collins *et al.* 1966).

Pritchard *et al.* (1978) have proposed the following atributes of an ideal vaccine:

(1). The vaccine requires strong protection against intestinal pressures such as gut pH.

(2). The vaccine should produce a prolonged protection.

(3). The mass administration of the vaccine should be possible ie. via water, food or oral spray.

(4). The yaccination should result in residual virulence transferable by vertical transmission.

(5). The vaccine should not reduce growth rate.

(6). The vaccine must be effective against infection at the time of immunization ie. confer immediate protection.

(7). The vaccine must have a degree of cross protection against other serotypes.

(8). The vaccine should contain a genetic marker to distinguish it from wild types.

(9). Vaccination should be compatible with and complementary to competitive exclusion, and antibiotics employed for growth promotion.
(10). The development of problems analogous to those of antibiotic resistance should be avoided.

Starvation Survival and Nutritional Immunity

In the work reviewed (p30-42) and summarized in Table 6, physiologically fit organisms were used. In practice the hen's egg may well be infected with physiologically damaged, phenotypically maladjusted or quiescent organisms.

Kochan (1977) found that the addition of iron or siderophores to the blood of animals promoted bacterial growth. This led him to define a novel aspect of the antimicrobial defence in animals, "nutritional immunity". He identified transferrin in blood, lactoferrin in milk and ovotransferrin in egg albumen as causative agents of this state.

Apart from ovotransferrin, egg white contains several other proteins (Table 5), avidin (chelation of biotin), ovomucoid (inhibition of trypsin), thiamin-binding protein and ovoflavoprotein (binds riboflavin), which could well contribute to nutritional immunity. The combination of these proteins may well induce contaminants of the albumen to enter the starvation-survival state.

All organisms, including bacteria, have one aim - they strive to retain viability in order to create progeny (Morita 1982) despite environmental stresses. The environment commonly imposed on bacteria in the laboratory has led to seven stages of bacterial life being identified: initial stationary, lag or growth acceleration, logarithmic growth, negative growth acceleration, stationary phase, accelerated death and logarithmic death (Buchanan 1918). Microbiology textbooks have condensed these into three or four stages, lag, exponential, stationary and the death or decline phase. The last mentioned is the most important one in starvation survival.

Growth is sporadic in the natural environment (Morita 1982), a period of unstable growth may be followed by starvation (Dawes 1984). Indeed in a natural environment nutrient-limited growth is probably the normal state (Colwell 1991). Consequently a major part of the total microbial biomass in a stressed environment will be made up of cells in a state of quiesence or suspended animation (Lewis and Gattie 1991). It is these organisms in the biological state of "no growth" (Morita 1982) rather than those grown under ideal laboratory conditions (Colwell 1991, Reeve, Bockman and Matin 1984, Morita 1988, Morita 1982) which can be considered to resemble the common cell condition (Novitsky and Morita 1976). Bacteria must, therefore, possess physiological or morphological attributes in order to survive such environmental stresses as pH extremes, desiccation,

nutrient limitation, adverse temperatures etc. (Schultz and Matin 1988). Classically the bacterial endospore or cyst was identified as adaptations to tide organisms over periods of stress (Hirsch *et al.* 1979). Currently it is beginning to be recognised that subtle ammendments of cell physiology and structure without gross changes in morphology confer protection on many organisms (Kjelleberg, Humphrey and Marshall 1983, Harder and Dijkhuizen 1983).

Starvation survival is vitally important as it provides a means of survival of the species in nature (Amy and Morita 1983). A single viable cell surviving in a stressed environment - ie. in the absence of an energy yielding substrate (Morita 1982), is all that is required for the expression of its genome when the environmental conditions become more favourable (Morita 1982, Amy and Morita 1983) and the perpetuation of the species (Sussman and Halverson 1966).

Every species of bacterium must have a low nutrient threshold. It dictates an organism's ability to remain viable or enter the quiescent/dormant state (Sussman and Halverson 1966). Organisms with a very low nutrient threshold will have a selective advantage through their ability to utilize scarce energy-yielding substrates when these become available (Dawes 1976). The microbial growth rate will be determined by the ability of an organism to transport the nutrient in greatest demand at an appropriate rate, or to use its reserve pools of that nutrient (Martin and Mcleod 1984). To achieve this

state, such organisms require effective transport systems and low maintenance energies (Koch 1979).

Microbial survival strategies

A population of cells may respond in one of four ways to the onset of starvation: numbers of viable cells may remain unchanged, the cells may increase in number before declining to a constant population size, cells may increase in number and then remain constant, or decrease in number to a lower population size (Amy, Pauling and Morita 1983, Morita 1985).

An initial increase in cells number during early starvation is accomplished usually by fractionation of existing cells into smaller ones ie. division without growth (Kjellberg and Hermasson 1984). Fractionation - miniturization or dwarfing - is a common response in many bacteria (Gottschal 1992). The formation of such cells results in an increased surface area to volume ratio. This may well aid the acquisition of substrates (Koch 1979, Morita 1982). Fractionation leads to continuous size reduction and, should adverse conditions persist, death (Kjelleberg *et al.* 1983). Marine organisms are notable for fragmentation (Torella and Morita 1981) and are commonly referred to as ultramicrocells (Novitsky and Morita 1976). Hood *et al.* (1986) noted that upon starvation, *Vibrio cholerae* cells became more coccoid, losing 90% of their original volume in 30 days. All granules and inclusion bodies were lost as well as the distinct 3-layered nature of the outer and cell

membranes. The nuclear region became condensed into the centre of the cell. The cell wall formed an extended or convoluted structure and the periplasmic space was enlarged. The ribosomal structures, however, exhibited no apparent change.

Koch (1979) states that there are two alternative strategies for the survival of organisms faced with starvation conditions. In one case an organism rapidly consumes the available resources and then moves to a new habitat, chemotaxis being important in this process (p69). In the second an organism becomes adapted to a situation where there is a continual renewal of resources at very low concentrations. These must therefore be utilized efficiently. For example *Caulobacter* exist in environments where both nutrient and total bacterial counts are low. They adapt by producing an elongated cell appendage - prostheca (Staley 1968). This is produced as an interuption to the cell cycle in low nutrient conditions. The cell cycle does not continue until the rate of nutrient uptake by the protheca signals the presence of sufficient nutrients to support its completion. Meanwhile cellular metabolism proceeds at a minimal rate (Poindexter 1979).

The onset of starvation may also initiate intense metabolic activity (Kjellberg *et al.* 1984). In practice protein synthesis is required to inactivate energy wasting enzymes and provide a pool of free amino acids (Reeve *et al.* 1984) in order to synthesize the enzymes required specifically for starvation conditions

(Mandelstam 1958) and to prolong viability (Schultz and Matin 1988). Rates of protein degradation and synthesis in starved cells are usually balanced. This would suggest that protein degradation is the major source of the pool of amino acids (Mandelstam 1958). For example, the onset of carbon starvation, induces the synthesis of approximately 30 proteins in *Escherichia coli*, many of which are not synthesized during normal growth (Groat *et al.* 1986). These are the enzymes involved in the capture of the required nutrient (Matin *et al.* 1989). The energy-yielding mechanism therefore remains intact in starved cells (Novitsky and Morita 1978).

The composition of the cell envelope may be altered also under different growth conditions (Lugtenberg *et al.* 1976). Scanning electron micrographs have shown an increase in surface roughness of starved cells (Kjelleberg and Hermasson 1984). Such changes in the components of the cell envelopes may affect cell physiology and eventual survival or death of the organism (Chai 1983). Thus in low nutrient environments it is essential that organisms have mechanisms for the uptake of solutes and ions. These systems must respond immediately to the sudden occurrence of nutrients. The integrity and functions of the cytoplasmic membrane, therefore, must be maintained during starvation (Dawes 1985).

Heat shock proteins

All organisms investigated to date are capable of responding to environmental stress by inducing the synthesis of a small set of proteins (Watson 1989). Jenkins, Schultz and Matin (1988) established that those induced by glucose or nitrogen starvation in *E.coli* also provided cross protection to heat and H_2O_2. The degree of resistance increased with the duration of cell starvation. Four hours provided the maximum protection. Thirty proteins were synthesized during glucose starvation and these provided resistance to heat or H_2O_2 also.

Microbial "heat shock" proteins may be induced by other environmental stresses such as ethanol, arsenite, heavy metals, amino-acid analogues, oxidative agents or anaerobiosis (Watson 1989). Many of these treatments are considered to induce the accumulation of damaged, deanilined or improperly folded proteins. It is the generation of these proteins caused by environmental stresses that has been proposed as the major trigger for the induction of "heat shock" proteins (Hightower 1980).

Cryptic growth

Survival may occur through the phenomenon of cryptic growth. That is a proportion of a population dies, lyses and releases nutrients to support the growth of others (Postgate 1976). This condition was first described by Ryan (1959). *Salmonella enteritidis* is able to grow on the products released by dead cells and has a relatively

long half life compared with other coliform organisms. This may result in significantly longer survival times *viz à viz* other coliforms or indicator organisms of faecal contamination in natural habitats, for example, soil or water (Druilhet and Sobek 1976).

To summarize, natural environments exert strong selective pressures. In order that the organism survives, it must evolve mechanisms to restrict damage to the cell and to ensure growth when the amount of growth-limiting substrate exceeds the limiting threshold (Veldkamp and Jannasch 1972). Thus the organism will need the ability to increase the rate of transport of a nutrient when its concentration becomes growth limiting. Indeed, the organism must increase the initial rate of metabolism of the accumulated nutrient when its intracellular concentration is low, and must have the ability to rearrange the chemical composition of its cellular structures by redirecting metabolites which contain the limiting nutrient (Harder and Dijkhuizen 1983).

Viable or non-viable

An early response to starvation is the loss of culturability on agar (Rozak and Colwell 1987) - Table 7. Viability has been defined as the "capacity to form colonies on agar medium suitable for the growth of organisms and non-viable when this ability is lost" (Colwell 1991). This is an unsatisfactory definition, however, as Postgate (1976) observed that bacteria may

Table 7. Selected characterisitics of viable but non-culturable bacteria*

Culture	1	No growth on or in standard culture media
	2	Resumption of growth under appropriate conditions
Cytology	1	Cells appear intact when stained by Acridine Orange and/or Antigen specific fluorochrome
	2	Cells are substrate responsive
	3	Cells may undergo changes in size (*viz* smaller) and/or shape (*viz* rounder)

* Grimes *et al.* (1986)

lose their ability to multiply but remain biologically functional entities. Thus an organism is viable if it is capable of reproduction in "optimal" conditions (Postgate, Crumpton and Hunter 1961). The loss of culturability is a reversible condition, unless starvation or some other stress persists (Rozak and Colwell 1987).

The time required for the initiation of cell growth is related directly to the length of the starvation period (Amy *et al.* 1983). Recovery is also dependent upon the number of viable cells remaining in a niche (Amy *et al.* 1983).

Substrate Accelerated Death

Growth of stressed organisms may be prevented by some component of a selective culture medium which would normally support unstressed organisms (Noble 1935). In fact "luxuriant" media may cause nutritional shock to organisms (Buck 1977). Postgate and Hunter (1963) state that a population exposed to a recovery medium "may yield dead cells and that this may be termed substrate accelerated death". That is growth substrates may accelerate the death of cells in a population from a low nutrient environment (Postgate & Hunter 1963). This phenomenon is due probably to the fact that survival in a stressed environment renders organisms hypersensitive to mild stresses and ones to which they would not normally react (Postgate and Hunter 1963). Clark & Ordal (1969) observed that, when compared with laboratory cultures,

injured *Salmonella* cells demonstrated a marked difference in their ability to grow on solid selective media normally used for their isolation and identification.

The effect of nutrients in the recovery medium depends upon the phase of growth during which the organisms were stressed, the type and intensity of stress, the growth condition before stress, the properties of the particular strain of organism involved and the cytological impact on injured populations (Mossel and Van Netten 1984).

It was suggested that the following be considered for the detection of stressed organisms. Damage to the cytoplasmic membrane may cause organisms to become temporarily susceptible to many selective compounds in media. Thus the cell must have a period in which to repair before exposure to selective pressures. It must be remembered, however, that a surviving population will contain uninjured cells. These will multiply first thereby masking the presence of stressed ones. Plating methods for indicator organisms can be seen therefore to have severe limitations (Rozak and Colwell 1987). This indicates the need for a more direct method to detect the presence of stressed micro-organisms (Colwell 1991). Indeed the difference between plate (viable) count and direct count has been widely noted (Table 8).

Various methods have been developed for the enumeration of viable organisms (Table 9). Early experiments demonstrated a difference between the number of living organisms and the total number of cells in a

Table 8. A history of the detection of higher numbers of cells by direct count than by plate count (various methods)

Reference		Method
1898	Winterberg	Thoma-Zeiss Chamber
1900	Klein	Aniline Gentian Violet
1901	Hehworth	" "
1906	Zelikow	Dye absorption/calorimeter
1932	Butkevich	Direct microscope counts
1938	Butkevich	" " "
1954	Wade & Morgan	Toludine Blue
1959	Jannasch & Jones	Methylene blue/membrane filter
1979	Hirsh *et al.*	" " "
1979	Kogure *et al.**	Nalidixic acid & Yeast extract
1983	Amy *et al.*	Epifluorescence
1986	Rollins & Colwell	Epifluorescence & Direct count
1991	Byrd *et al.*	Acridine Orange/Kogure method*

Table 9. Methods used in direct viable counts

Method	Variation	Reference
Plating,Miles & Misra Roll tubes, MPN		Postgate (1961)
Slide Incubation	Annulus and agar	Postgate & Hunter (1963) Zaske *et al.* (1980)
	Cellophane on agar	Powell (1956)
Nalidixic acid & Yeast extract	Seawater sample	Kogure *et al.* (1979)
Acridine Orange/ Nucleopore filters	Natural waters	Hobbie (1977) Francisco *et al.* (1973)
Methylene Blue	Yeast viability	Lee *et al.* (1981) Vairo (1961)
Toludine Blue	Vital stain	Wade & Morgan (1954)
Epifluorescent Microscopy	Fluorescent antibody	Xu *et al.*(1982) Amy *et al.*(1983)
Malachite Green-INT	Active bacteria	Dutton *et al.* (1983)
Trypan Blue	Vital stain	Pappenheimer (1917)
	Tumour cells	Hoskin *et al.* (1956)
	Plant Nuclei	Gurr (1965)
	Fish cells	Holl (1965)
	Mouse cells	Sawicki *et al.* (1967)
	Gastric cells	Brus & Glass (1973)
	Bacterial cell viability	Dereniz & Schechtman (1973)
	Bacterial cell viability	Phillips (1973)
	Bacterial cell viability	Anon (1991)

broth culture (Wilson 1922). For example, Klein (1900) appears to have been one of the first to realize the importance of the direct count in this context. This form of detection (direct count microscopy) has led to counts 200 to 5,000 times higher than plate counts (Butkevich 1932). It was concluded that a proportion of the bacteria must have been present in the resting stage (Butkevich and Butkevich 1936).

This review has presented an outline of conditions that might induce the formation of *Salmonella* somnicells. The influence of starvation on the behaviour of *S.enteritidis* and its somnicells in albumen from hens' eggs has never been discussed in the literature. The present study sought to remedy this omission.

Chemotaxis in the hen's egg

When the contents of a hen's egg become contaminated oviducally or by the trans-shell route, the organisms may persist but not multiply in the albumen for upwards of 2-3 weeks (Brooks 1960, Table 6). During this period changes in yolk structure may lead to the loss of nutrients to the albumen (Humphrey 1991). Some workers have contended that contaminants may grow as a consequence of such addition of nutrients "negating the inhibitory properties of the albumen" (Humphrey 1991). Another senario is worthy of consideration also, namely that loss of nutrients from the yolk establishes a

gradient that induces a chemotactic response in the contaminants.

To defer entry into a quiescent state, an organism may exploit chemotaxis to seek nutrients in a new niche. Chemotaxis is the movement of an organism towards or away from a chemical substance (Hazelbaur and Parkinson 1977). Initial studies (Englemann 1883, Pfeffer 1884, 1888, 1904) led to the conclusion that the attraction of bacteria to specific compounds was the result of the particular qualities of the attractant. Primarily chemotaxis serves 2 functions: as a food-seeking mechanism (Alder 1969), and a method for avoiding toxic or unfavourable environments (Hazelbaur and Parkinson 1977). Alder (1969) developed an assay based on the work of Pfeffer (1884,1888). This quantitative assay technique measured the degree of chemotaxis by the accumulation of organisms in a capillary containing an attractant. The bacteria in the tube were enumerated by the plate count method.

Bacterial chemotaxis occurs as a 2 step process: (1) the detection of changes in concentration of an appropriate compound, and (2) the modulation of the tumble generator.

In the first instance chemosensors monitor gradients. This requires that the organism detects the presence of a compound and determines its concentration relative to previous environments (Hazelbaur and Parkinson 1977). Chemotaxis operates over a limited range of concentrations - the "response range" (Mesibov,

Ordal and Alder 1973) - which extend from the "threshold concentration" - the lowest concentration to give a response, and the "saturating concentration" - the level beyond which bacteria cannot detect any higher concentrations (Alder 1973). It would appear that chemoreceptors are located on the outside of the cell (Alder 1969). Organic chemicals such as amino acids, oligopeptides, sugars and carboxylic acids (MacNab 1976) act by binding to specific receptors and initiating a process particular to that chemical. These signals emanate from the reversible methylation of accepting receptors/transducers of the membrane, as a result of external binding of the attractant (MacNab 1976). The result is a conformational change at an allosteric binding site. The receptor then transmits information about its conformation through association with another macromolecular component in the membrane (MacNab 1978).

Six soluble proteins (Che) are required for chemotactic signalling in *S.typhimurium*. CheR and CheB are involved in signal adaptation of the methyl-accepting chemotaxis proteins, and CheA, CheW, CheY and CheZ, which are necessary for integrating and transmitting the signal from the receptors to the flagella motor (Matsumara *et al.* 1990). *Salmonella typhimurium* possesses 3 cardinal receptors or interacting receptors for amino acids - for aspartate, serine and alanine classes (Melton *et al.* 1978), with the most attractive amino acids being those which can act as a sole carbon source (Bochner and Saveageau 1977). Repellents induce responses at higher

concentrations than attractants (MacNab 1976). Changes in the occupancy of the receptor site can be related directly to the swimming behaviour (Berg and Tedesco 1975).

In the absence of stimuli, bacteria swim in a 3-D random pattern (Berg and Brown 1972). Tumbling is a random event. Bacteria moving up a gradient exhibit greater mean run lengths (ie. less tumbles) than those not exposed to a gradient. Thus chemotactic migration is achieved by the control of the run length, rather than the velocity of the organism (Hazelbaur and Parkinson 1977). The tumbling frequency is moderated by a switch which controls whether or not the flagella rotate counter-clockwise - for swimming, or clockwise - for tumbling (Ordal 1976). The tumbling frequency is the most important factor in the tactic response of bacteria (Meada *et al.* 1976).

A divalent cation, such as Mg^{2+} bound to the above noted switch, causes swimming. In its absence the bacterium tumbles. Increased tumbling caused by repellents may hinder access of Mg^{2+} to the switch. Attractants would seem therefore to cause the increased access of Mg^{2+} to the switch (Ordal 1976).

Tumbling frequency is affected by many temperature-dependent factors. For example, the change in fluidity of *E.coli* cell membrane caused by altering lipid composition did not affect the temperature dependence or swimming speed of the organisms, but resulted in a drastic change in the temperature dependency of the

chemotactic response. Generally the swimming velocity of *E.coli* was found to increase with increasing temperature due to the tumbling frequency peaking at 34 ^{0}C over a range of 20 to 39 ^{0}C. A temperature drop increased the tumbling frequency and a temperature increase, decreased the frequency (Meada *et al.* 1976).

Regarding the possibility of chemotaxis in the hen's egg, a fertile chicken egg shows a higher concentration of amino acids in the yolk than in the albumen. Ducay, Kline and Mandeles (1960) found a total of 16 amino acids occurring in both white and yolk. A gradient, however, is maintained across the vitelline membrane, which in isolation is permeable to the amino acids alanine, leucine and phenylalanine. The gradient is maintained because amino acids are bound to protein on both sides of the vitelline membrane in order to ensure optimal concentration for the developing embryo (Pons *et al.* 1985). It can be assumed therefore that the vitelline membrane constitutes no barrier to the diffusion of amino acids between yolk and albumen (Säinz *et al.* 1983). Recent studies by Dolman and Board (unpublished) have failed, however, to show any correlation between increasing amino acid levels in the albumen and microbial growth. In the present study I sought to establish whether or not a chemotactic response played a part in the process leading to a generalized infection of an egg's contents.

SECTION 1

A study of the behaviour of *Salmonella* serotypes in albumen

This, the first section of results, deals with the fate of serotypes and phage types of *Salmonella* in albumen *in vitro*. As was noted in the Literature Review, there is much circumstantial evidence (Table 6) to the effect that rot producing bacteria that pass from the shell membranes to the albumen remain quiescent until freed from the immediate influence of the antimicrobial agents of the albumen. The literature would lead us to believe that, in most cases, the collision of contaminants and yolk results in this state. Previous studies of albumen *in vitro* (Board and Halls 1973, Sparks and Board 1985) have shown that ferric ammonium citrate or combinations of simple nitrogenous substances and iron negates the antimicrobial properties of the albumen.

As the recent study by Clay and Board (1991) indicated that salmonellas behave in much the same manner as rot-producing bacteria in whole eggs, this part of the study sought information about the behaviour of the former during the quiescent phase (Table 6) of infection of eggs.

MATERIALS AND METHODS

Eggs

Eggs (size 4, approx 58g) less than 2-days old were purchased from a local producer/retailer and stored for less than 2 days before use. Eggs were assumed to be salmonella-free at purchase. Eggs from the same source were used in other studies in which endogenous salmonellas would have been detected. None was found to be contaminated.

Cultures

The sources of the cultures are given in Table 10 These were stored on Dorset egg agar (Oxoid Ltd) at 4 ^{0}C and subcultured every 3 months. For experimental purposes, an overnight culture (18 h) in nutrient broth (Lab M, incubated at 37 ^{0}C) was spun down (2000 g for 10 min). Washed in saline (0.85% w/v, Lab M), and finally resuspended in the same medium and diluted such that 0.1 ml contained ca. 10^{3} organisms. Aged cultures were prepared by extended incubation at 4, 20 or 37 ^{0}C.

Presumptive *Salmonella* colonies were confirmed serologically (Wellcome Diagnostics).

Persistence of various *Salmonella* spp. in albumen *in vitro*

Eggshells were wiped with ethanol (70% v/v), cracked and the contents collected. Albumen and yolk were harvested and bulked separately. Seventy ml of blended albumen were dispensed into sterile specimen pots (250 ml, Sterilin) and 0.1 ml of a bacterial suspension added. Duplicate samples were stored at 4, 20 or 30 ^{0}C and sampled frequently. Ferric

ammonium citrate (BDH) was added to the albumen (final concentration 0.008 mg/ml Fe^{3+}) on the 42^{nd} day of incubation.

Viable counts were obtained by spreading 0.1 ml of an appropriate dilution on duplicate plates of Xylose Lysine Decarboxylase agar (XLD:Lab M) with overnight incubation at 37 ^{0}C. A comparison of viable counts on XLD and Nutrient agar for the isolation of *Salmonella* from albumen was carried out. Presumptive *Salmonella* colonies were confirmed serologically. Initial and final glucose concentrations were determined (Boehringer Mannheim - W.Germany). The pH of the albumen was tested with Whatman indicator paper (range 1-14). Laboratory safety regulations precluded the use of a pH meter with material contaminated with *Salmonella*.

The following variations to the experimental plan were done.

(a). Determination of the effect of inoculum size on the response of *S.enteritidis*, *S.pullorum*, and *S.hadar* in albumen *in vitro*

Albumen was bulked and dispensed into 250 ml specimen pots (Sterilin). An overnight cell suspension (0.1 ml) was added such that the albumen contained ca. 10, 10^3 or 10^6 *S.pullorum*/ml or *S.hadar*/ml and 10, 10^2, 10^3, 10^4 or 10^6 *S.enteritidis*. Duplicate samples were stored at 4, 20 or 30 ^{0}C. Viable counts were obtained on XLD. The effect of the addition of ferric ammonium citrate (final concentration 0.008 mg/ml Fe^{3+}) on the 42^{nd} day was noted. The pH of the albumen was tested with Whatman indicator paper (range 1-14).

(b). The study of the effect of various nutritional additions on the behaviour of *S.enteritidis* in albumen *in vitro*

Various nutritional additions were made to albumen (70 ml) containing ca. 10^3 organisms/ml;

1). Ferric ammonium citrate - 0.008 mg/ml.

2). Faecal extract - An extract of adult chicken faeces was prepared by diluting faeces 1 in 10 in water (wet weight) and filtering twice through muslin. One ml of the sterilized filtrate was added to the albumen.

3). Iron-reduced faecal extract (IEFE) - Faecal extract was prepared as above and the iron extracted by the method of Waring and Werkman (1942). The faecal extract was poured into a glass stoppered separating funnel (acid washed). About 5 mg of crystalline 8-hydroxyquinoline (Fisons) was dissolved in 1 ml of chloroform (BDH) and poured into the separating funnel, which was then shaken vigourously before being allowed to stand for 5 min. About 3 ml of chloroform were added to the funnel which was shaken vigourously for one minute, and rotated for one minute to allow the chloroform to coalesce at the base of the funnel. The chloroform was drawn off and the solution washed twice with further 3 ml portions of chloroform. The whole extraction process was repeated twice before a final rinse with 5 ml chloroform. The final preparation was transferred to an acid-washed flask, and sealed with a glass beaker.

4). Nitrogen source - NH_4Cl (Fisons) was filter sterilized (0.45 µm: Oxoid Ltd) and added to the albumen to give a final concentration of 1 mg/ml.

5). Growth factor solution (GF) - consisted of (mg/ml distilled water) *p*-aminobenzoic acid (Sigma), 10; folic acid (BDH), 1.0; vitamin B_{12} (Sigma), 1.0; nicotinic acid (Sigma), 1.0; pantothenic acid (BDH), 1.0; thiamine (Sigma), 1.0; riboflavin (Sigma), 1.0 and biotin (Aldrich), 1.0. One ml of filter-sterilized solution (0.45 µm: Oxoid Ltd) was added to the albumen (Tranter 1982).

Initial and final glucose concentrations were determined (Boehringer Mannheim) - see below. The pH of the albumen was tested with Whatman indicator paper (range 1-14).

Glassware

All glassware used in experiments involving iron was freed of contaminating iron by the following procedure. The glassware was soaked in 0.1M HCl (BDH) for 14 h and rinsed in de-ionised, glass distilled water. This was followed by a wash in 8-hydroxyquinoline (0.5% w/v) in chloroform (rinsing in chloroform), prior to drying in a hot air oven. The glassware was then rinsed twice in de-ionised glass distilled water and dried in a hot air oven. Washings from the glassware contained mimimal amount of iron (<0.1 µg/ml).

Chemical determinations

Glucose. Glucose concentrations were determined using the Boehringer blood sugar kit (Boehringer-Mannheim). A sample of albumen (0.1 ml) was precipitated in 1.0 ml (0.16% w/v) uranyl acetate (Tabb Laboratories). The protein was pelleted by centrifugation (5,000 g x 10 min) and 0.2 ml of the supernatant used for the assay. Glucose oxidase reagent

(5 ml); peroxidase, 20 µg; glucose oxidase, 180 µg; chromatogen (ABTS), 0.5 mg in 100mM phosphate buffer (pH 7.0) were added to the supernatant, a glucose standard and the distilled water blank. The mixture was incubated at 37 ^{0}C for 20 min and the absorbance measured at 610 nm by a Unicam Sp500 spectrophotometer (Pye-Unicam Ltd).

Glucose concentration = $\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$ (mg/100 ml)

Iron. The iron concentrations of faecal extract and iron-reduced faecal extract were determined by the phenanthroline method (Anon 1975).

1). Calibration - A calibration range of 0-500 µg Fe/100 ml was prepared. Known volumes of a standard iron solution (Appendix 1) were pipetted into 100 ml volumetric flasks. To this were added: hydoxylamine solution (Sigma) - 1.0 ml, and sodium acetate solution (Sigma) - 1.0 ml (Appendix 1). The volume was then made up to ca. 75 ml with de-ionised water. Phenanthroline solution (10 ml) was added prior to it being diluted to volume. The solution was allowed to stand for 10 min. Absorbance was measured at 508 nm in 1 cm cells and a calibration curve prepared.

2). Total iron determination. A known sample volume was dispensed in a fused silica boat (acid washed) and allowed to evaporate in a steam bath until a suitable volume decrease had occurred. A minimum volume of concentrated HCl and warm de-ionised water were added. The mixture was filtered and neutralised with NH_4OH (FSA) before being made up to volume (50 ml). To this mixture were added

concentrated HCl, 2 ml; hydoxylamine solution, 1 ml, and glass beads (Appendix 1). The volume of the mixture was reduced to 15-20 ml by boiling. After cooling to room temperature it was transferred to a volumetric flask (100 ml) and the following added: ammonium acetate (BDH) buffer solution, 10 ml; phenanthroline solution, 2 ml, and de-ionised water to dilute to 100 ml. The solutions were mixed thoroughly and allowed to stand for 10-15 min. Absorbance at 508 nm was measured.

(c). The determination of the duration of motility of synchronous and non-synchronous populations of *S.enteritidis* in albumen *in vitro* and the effect of various nutritional supplements

Synchronous cultures were prepared as described by Cutler and Evans (1966). *Salmonella enteritidis* was grown overnight in nutrient broth (Lab M - incubated at 37 ^{0}C), the cells were harvested in early stationary phase (pre-determined by a growth curve) by centrifugation and diluted 7-fold into fresh medium. This took place in the minimum length of time possible. The cells were re-incubated and harvested in early stationary phase and the process repeated. The cells were assumed to be synchromous and finally resuspended in saline (0.85%, w/v, BDH) and diluted such that 0.1 ml contained the required number of cells.

An overnight culture (non-synchronous) in nutrient broth was washed in saline (x2) as described above (p75). Albumen was dispensed into sterile containers (Sterilin 250 ml) and 0.1 ml of cell suspension added. Various

nutritional supplements were added to the albumen (as outlined above). The albumen was stored at 4 or 25 ^{0}C.

A motility index of organisms in albumen was obtained by comparing the number of motile and non-motile cells. A minimum of 200 cells were counted per sample using x1000 phase contrast microscopy (Olympus BH2).

Addition to albumen of *S.enteritidis* and antibodies raised in chickens

The anti-serum was raised at Langford Veterinary School (Janet Bale), with the following procedure;

(1) Preparation of *Salmonella* "OH" antiserum.

(a) A well isolated "smooth" colony was selected from a plate culture.

(b) The antigenic structure was confirmed by a slide agglutination test.

(c) A portion of the checked colony was inoculated into a Craigie tube, which was incubated at 37 ^{0}C until bacterial growth was visible on the surface of the medium in the outer tube. This growth was used to inoculate another Craigie tube.

(d) The procedure was repeated 4 times, each time the culture was checked for hyper-motility by the "hanging-drop" method.

(e) Pre-warmed peptone broth (Lab M, 10 ml) was inoculated with the hyper-motile culture and incubated at 37 ^{0}C for 60-90 min, before being added to 500 ml of pre-warmed peptone

broth. This was incubated at 37 ^{0}C for 6-8 h.

(f) The cell suspension was checked for hyper-motility before 0.3% (w/v) formaldehyde (BDH) was added. The solution was allowed to stand overnight.

(g) The suspension was inoculated in a marginal vein (eg. wing), at 3-4 day intervals with incremental doses of immunogen, 0.1, 0.2, 0.4, 0.8, 1.6 ml. One week after the final inoculation, a test bleed was performed.

(2) Bleeding. A test bleed was taken initially from the wing vein and later collections of larger samples taken from the brachial vein.

(3) Isolation of the serum from blood. Serum should be separated from the cells as soon as possible after collection of the blood, to prevent the lysis of cells and release of proteins which include proteases capable of degrading antibodies.

(a) The blood was allowed to clot at room temperature for 1 h before being left overnight at 4 ^{0}C.

(b) The clot-free liquid was poured into a centrifuge tube and kept at 4 ^{0}C.

(c) The clot was centrifuged (2500 g) for 30 min at 4 ^{0}C and any liquid present removed and added to tube (b).

(d) The pooled liquid was centrifuged (1500 g) for 15-20 min at 4 ^{0}C. The serum was stored at 4 ^{0}C. Note: chicken serum must not be frozen.

(4) The antibody titre was determined using the Widal method (Anon 1960).

Seven tubes were placed in a rack and labelled as follows;

(1)	(2)	(3)	(4)	(5)	(6)	(7)
1/15	1/30	1/60	1/120	1/240	1/480	control

To tubes 2-7, 0.4 ml of saline was added; to a separate tube, 0.1 ml of the serum was added to 1.4 ml saline (1/15 dilution). To tubes (1) and (2) 0.4 ml of the 1/15 dilution were added, thus making a 1/30 dilution. Following this, 0.4 ml of tube (2) was added to tube (3), thus making tube (3) a 1/60 dilution. The process was continued to tube (6) from which 0.4 ml was discarded. Thus tube (7) contained saline only. Bacterial suspension (0.4 ml) was added to each tube giving the following dilutions:

(1)	(2)	(3)	(4)	(5)	(6)	(7)
1/30	1/60	1/120	1/240	1/480	1/960	control

Clumping was observed in tubes after 2 h at 50-55 ^{0}C.

(5) To dispensed whole albumen (70 ml) was added serum (0.15 ml of a known titre) and *S.enteritidis* suspension such that the albumen contained ca. 10^{3-4} or 10^{6-7} cfu/ml. Viable counts were made on XLD and microscopic observations done. The albumen was stored at room temperature (ca. 22-25 ^{0}C).

Experimental variation included;

(a) Using thin albumen only.

(b) Including complement. Complement was removed by heating at 55 ^{0}C for 30 min.

(c) Using Ringers solution instead of albumen to

determine if the albumen's structure encouraged clumping.

(d) Using an aged (stored for >7 days at 30 ^{0}C) culture *S.enteritidis*.

The motility of the organism was observed throughout these procedures.

RESULTS

The persistence of *Salmonella* in albumen *in vitro*

Earlier studies of albumen *in vitro* have shown that combinations of simple nitrogenous substances and iron negate the antimicrobial properties of the albumen (Sparks and Board 1985). This was confirmed in the present study (Figure 4). It was also noted by these workers that growth induced by the addition of ferric ammonium citrate was associated with the depletion of glucose and an acid shift in the pH (9.5 to 7.0-8.0) of the albumen. This was also confirmed in the present study (Figure 5). As ferric ammonium citrate overcame the antimicrobial attributes of albumen it was used routinely in the present study.

The persistence of viable cells (initial inoculum ca. 10^{3}-10^{4} cfu/ml) in albumen *in vitro* at 4, 20 or 30 ^{0}C was studied with 13 *Salmonella* serotypes and 13 phage types of *Salmonella enteritidis* (Table 10). With the exceptions of *S.enteritidis* (Figure 6) and *S.hadar*, and both on one occasion only, there was a progressive diminution in the

viable counts in albumen at 4 ^{0}C such that our method of analysis failed (<10 cfu/ml) to isolate organisms on the 42nd day of incubation. As was to be expected the addition of ferric ammonium citrate to albumen on the 42nd day of incubation did not induce multiplication at 4 ^{0}C of any of the *Salmonella* serotypes or phage types.

There was a spectrum of responses among salmonellas stored in unsupplemented albumen incubated at 20 or 30 ^{0}C. Six of the 13 phage types of *S.enteritidis* (Table 10) multiplied sluggishly (generation time of days) at one or both of these temperatures (Figure 7). When sluggish growth occurred neither the glucose content nor the pH of the albumen changed. It is evident from Figure 6 that there was a very fast rate of growth following the addition of ferric ammonium citrate to albumen at 20 or 30 ^{0}C. The pH of the albumen changed (pH 9.5 to 7.0-8.0) when the population of salmonellas attained >10^{7} cfu/ml. Likewise the concentration of glucose in the albumen diminished (50-100%) only when heavy growth occurred (Figures 4 & 5). The addition of other supplements, NH_4Cl or growth factor to albumen at 25 ^{0}C led to a large increase in population size when ferric ammonium citrate was present (Figure 4). These supplements produced no increase in cell numbers when added alone. The greatest increase in population size was achieved with the addition of all 3 supplements. Faecal extract and ferric ammonium citrate gave a comparably large increase in population sizes. The percentage of glucose remaining in the albumen appeared to be influenced by the combination of supplements. With NH_4Cl plus ferric ammonium

citrate and ferric ammonium citate alone, no glucose remained after 48 h. When ferric ammonium citrate was combined with growth factor, however, there was still <40% of the glucose remaining. The reasons for this are not known. The addition of faecal extract did not change the glucose concentration of the albumen despite inducing a large increase in population size. This suggests that the faecal extract contained sufficient nutrients for the growth of *S.enteritidis.*

The other phage types of *S.enteritidis* merely persisted ie. cell numbers either remained constant or diminished very slowly with time in albumen at 20 or 30 ^{0}C. There was no demonstrable change in the glucose content or pH of the albumen. Of the other serotypes only *S.hadar* (Figure 8), *worthinghton* (Figure 9), *ohio* (Figure 10) and *waycross* (Figure 11) multiplied in albumen at 20 or 30 ^{0}C. Again, however, the growth rate was sluggish and the results inconsistent. Thus, for example, *S.hadar* merely persisted in 2 trials and grew in another (Table 10). In the latter instance the generation time was very long (>10 days) in albumen at 20 ^{0}C. The number of viable cells of *S.pullorum* diminshed to undetectable levels at both of these temperatures.

Every strain of *Salmonella* (Table 10) used in this study formed large populations (>1 x 10^{7} cfu/ml) at 20 and 30 ^{0}C following the addition of ferric ammonium citrate to the albumen (Table 10). It needs to be stressed that the generation times (hours) in supplemented albumen were very

short *viz à viz* those of *S.hadar*, for example, in unsupplemented albumen.

Three of the organisms studied above, *S.enteritidis*, *S.hadar* and *S.pullorum*, were used in investigations which sought to answer the question: does the initial inoculum size influence the fate of salmonellas in albumen *in vitro* when stored at 4, 20 or 30 ^{0}C ? When 10 or 100 cells of *S.enteritidis* (Figures 12a & b) were added per ml of albumen, these methods failed to isolate any organisms throughout the 42 days of incubation. Nevertheless the addition of ferric ammonium citrate caused a rapid multiplication of the very few organisms in albumen incubated at 20 or 30 ^{0}C but not at 4 ^{0}C. An inoculum intended to give 1.0 x 10^{3} cfu/ml of albumen gave results (Figure 12c) analogous to those discussed above (Figure 6). With an inoculum of >1.0 x 10^{5} to <1.0 x 10^{6} cfu/ml (Figure 12d) there was a slow but progressive growth at 30 ^{0}C, but little growth at 20 ^{0}C. As would be expected, no growth occurred at 4 ^{0}C even following the addition of ferric ammonium citrate. As judged from the main survey (Table 10), in which the starting inoculum was 10^{3} to 10^{4} cfu/ml (Figure 6), results analogous to those discussed above were obtained with *S.hadar* (Figure 13a). With a higher inoculum of ca. 10^{6} cfu/ml, analogous results to those obtained with *S.enteritidis* (Figure 12d) were obtained at 4, 20 and 30 ^{0}C (Figure 13b). It is evident that of all the serotypes in this work *S.pullorum* was probably the the most poorly adapted from the point of view of persistence in albumen *in vitro*. Even with an inoculum of ca. 1.0 x 10^{6} *S.pullorum*/ml

(Figure 14b), the number of viable organisms diminished during incubation, with the fastest rate of death occurring at 30 ^{0}C. Growth occurred, however, following the addition of ferric ammonium citrate at 20 or 30 ^{0}C. At the lower inoculum level of ca. 10^{3} cfu/ml, the number of organisms had diminished below detectable levels by day 42. The addition of ferric ammonium citrate failed to induce growth at 4 or 20 ^{0}C (Figure 14a).

Microscopical examination (a minimum of 200 cells/ sample), which was done on every sampling occasion in one experiment with inoculated albumen at 4 or 25 ^{0}C, compared the number of motile and non-motile cells. It was found that some cells of *S.enteritidis* remained motile in albumen for between 2 and 20 days at 4 ^{0}C. When suspended in unsupplemented albumen at 25 ^{0}C, *S.enteritidis* PT 4 cells were motile on the 42nd day of storage (Table 11). The proportion of cells remaining motile at 4 or 25 ^{0}C in albumen after 5 days with various nutritional additions is shown in Table 12. It is evident from the Table that the proportion of motile *S.enteritidis* PT 4 cells was highest in albumen containing faecal extract. Upto 60% of cells were motile at 4 ^{0}C and upto 85% at 25 ^{0}C after 5 days incubation. Analogous results were obtained in albumen supplemented with ferric ammonium citrate. A higher percentage of cells remained motile at 25 ^{0}C than at 4 ^{0}C in supplemented and unsupplemented albumen. *Salmonella hadar* in unsupplemented albumen showed a higher percentage of cells remaining motile after 5 days (4 ^{0}C - 65%; 25 ^{0}C - 97%) than *S.enteritidis* (4 ^{0}C - 0-22%; 25 ^{0}C - 27-55%). The

motility of *S.enteritidis* PT 4 was noted also in "aged" albumen (7 days at 30 ^{0}C - before incubation). After 5 days, 14-55% of the organisms were motile at 25 ^{0}C, and 0-10% at 4 ^{0}C (Table 12). It was noted also that motility in synchronous and non-synchronous cultures gave comparable results. Although there was a range in the proportion of motile cells in albumen supplemented with various materials, it is evident that even with albumen alone motility of some cells persisted. The practical implications of this observation became evident when the possible role of chemotaxis in egg infection was investigated (p135).

The survival of *S.enteritidis* in albumen *in vitro* containing faecal extract was studied at 4, 20 and 30 ^{0}C (Figure 15). The pattern of growth at 20 and 30 ^{0}C showed a steady increase in numbers (10^{3} to 10^{8} cfu/ml) in albumen supplemented with faecal extract or iron-reduced faecal extract (IEFE). At 4 ^{0}C there was a steady diminution in numbers of viable organisms over the 14 days such that low or undetectable numbers were present by day 14.

The iron content of faecal extract and IEFE was determined using the phenanthroline method (Anon 1975). It was found that the iron content of IEFE (2.76 µg/ml) was just over half that of faecal extract (5.15 µg/ml). There was, however, no difference in the glucose concentrations (0.3 mg/ml).

In the work discussed above, emphasis was given to the chemical defence of the albumen. According to the literature (Kramer and Cho 1970), antibodies occur in the albumen. This section sought to determine whether or not

antibodies influenced the fate of *S.enteritidis* in albumen *in vitro*.

The addition of antiserum (0.15 ml of 1/240 titre solution) to blended albumen (70 ml) had no apparent influence on the feeble growth of the organism (Figure 16). When inoculated thick albumen was viewed microscopically, ca. 10-20% of the cells occurred in chains whether or not the albumen was supplemented with antisera containing complement. Chains were also evident, but much less frequently, in albumen alone infected with *S.enteritidis*. These studies were extended to include thin albumen. Again the organism (10-20%) formed chains. Thus to investigate the possibility of antisera alone influencing cell behaviour, Ringers solution supplemented with antisera was used. The same results were obtained, namely some cells formed chains. The presence or absence of complement appeared to have no effect on chain formation or motiltity. The latter observation showed that antisera alone caused organisms to form chains in albumen.

With another sample of complement-free antisera, it became apparent that antibodies only restrained growth in albumen diluted to a small extent with Ringers containing antibodies. In other words, cell viability decreased with increasing antisera concentration. It was evident that the antimicrobial effect of albumen together with the antisera (diluted to a minimal extent with albumen) hindered the growth of *S.enteritidis* compared with that in Ringers solution (Figure 17).

The motility of the organism ceased only in albumen when neat serum and *S.enteritidis* (ca. 10^8 cfu/ml) mixed in equal volumes were added. Generally cells in chains were less motile than solitary ones. This effect was more pronounced when an "aged" culture (7 days at 30 ^{0}C) was used. Large clumps were observed in this instance. In summary therefore antisera would appear to have little effect on the fate of salmonellas in albumen *in vitro*, unless extremely large concentrations of antibody are present.

Summary

In summary a study was made of the persistence of different *Salmonella* serotypes in hens' egg albumen *in vitro* at 4, 20 and 30 ^{0}C. The majority of serotypes studied remained viable but did not increase in number at 20 and 30 ^{0}C for 42 days. At 4 ^{0}C many serotypes died out.

The addition of ferric ammonium citrate on the 42nd day of incubation induced multiplication of organisms incubated at 20 and 30 ^{0}C, but not at 4 ^{0}C. The pH and glucose concentration of the albumen diminished only when heavy growth occurred.

Thirty percent of the organisms also remained motile in albumen for 42 days at 25 ^{0}C and up to 5% of the cells remained motile for up to 20 days at 4 ^{0}C.

The addition of *S.enteritidis* antibody to albumen containing *S.enteritidis* had no appreciable effect on multiplication or motility of the organism.

Figures 4 & 5.

Various nutritional supplements were made to albumen (70 ml) containing ca. 10^2-10^3 cfu/ml. The experiment was done in duplicate.

Final concentration of nutritional supplements in albumen:

NH_4Cl, 1 mg/ml

Ferric ammonium citrate (FAC), 0.008 mg/ml

Growth factor solution (GF), (mg/ml) *p*-aminobenzoic acid, 10; folic acid, 1.0; vitamin B_{12}, 1.0; nicotinic acid, 1.0; pantothenic acid, 1.0; thiamine, 1.0; biotin, 1.0; 1 ml added to albumen

Faecal extract, 1/10 dilution of faeces was made, of which 1 ml of sterilized solution was added to albumen

Figure 4 The fate of *S.enteritidis* in albumen plus additions in *vitro* at 25°C

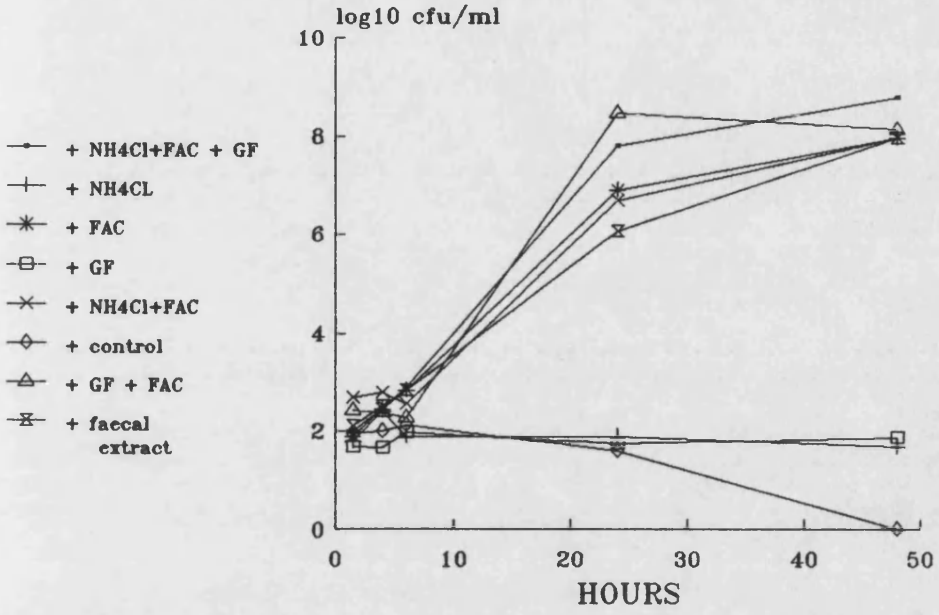


Figure 5 The percentage of glucose remaining in albumen *in vitro* inoculated with *S.enteritidis* at 25°C

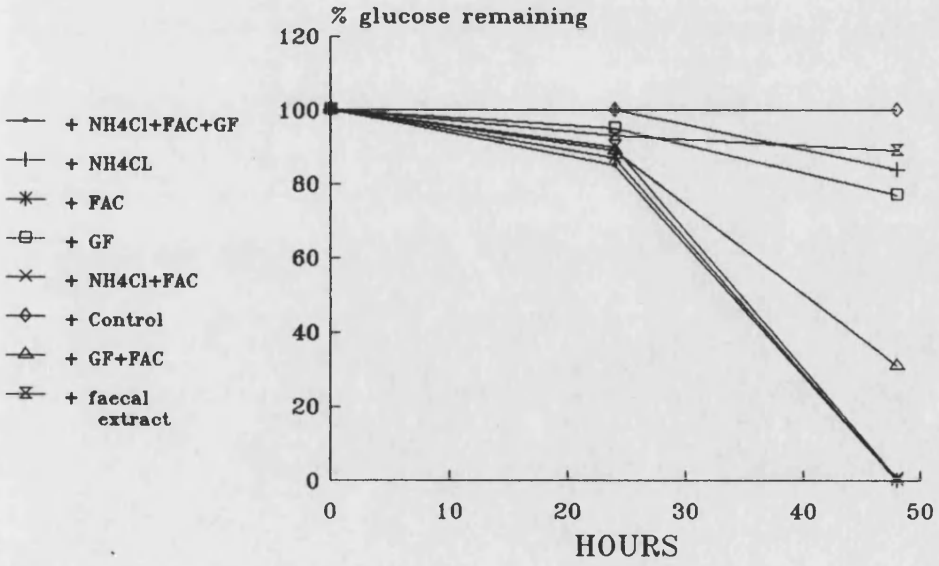


Table 10. The persistence of *Salmonella* serotypes in albumen *in vitro* with incubation at 4, 20 or 30 ^{0}C

Salmonella serotype	Source	Persistence at ^{0}C 4	20	30	Response to Fe^{3+} 4	20	30
enteritidis	A2*	+	+	+	ng	G	G
PT 4	A	-	g	g	ng	G	G
PT 4 cured	A	-	+	g	ng	G	G
PT 4a	B	-	g	(g)	ng	G	G
PT 1	B	-	g	g	ng	G	G
PT 6	B	-	g	(g)	ng	G	G
PT 13a	C	-	g	(g)	ng	G	G
PT 21	B	-	g	(g)	ng	G	G
PT 30	B	-	(g)	g	ng	G	G
PT 14b	B	-	(g)	(g)	ng	G	G
PT 5	B	-	(g)	(g)	ng	G	G
PT 12	B	-	(g)	+	ng	G	G
PT 8	C	-	(g)	-	ng	G	G
PT 24	B	-	+	+	ng	G	G
PT 23	B	-	+	-	ng	G	G
hadar	D2	+	+	(g)	ng	G	G
		-	(g)	(g)	ng	G	
worthington	D	-	g	(g)	ng	G	G
waycross	E	-	(g)	+	ng	G	G
ohio	D	-	(g)	+	ng	G	G
brandenberg	E	-	+	+	ng	G	G
dublin	E	-	+	-	ng	G	G
infantis	E	-	+	-	ng	G	G
typhimurium	A	-	+	+	ng	G	G
montevideo	B	-	+	+	ng	G	G
senftenberg	D	-	+	+	ng	G	G
gallinarum	F	-	+	-	ng	G	G
pullorum	F	-	-	-	ng	G	G

Source; A,Exeter PHLS; B,CVL (Weybridge); C,ex-egg USA; D,British United Turkeys; E,Bath University; F,Bristol University

-,did not persist; +,persisted; ng, no growth; (g),slight growth - generation time 10 -19 days; g, growth, generation time 2 -12 days; G, generation time 6 - 12 hours.

* all experiments done in duplicate , 2 indicates that 2 or more trials were conducted.

Figure 6 Persistence of *S.enteritidis* in albumen *in vitro*

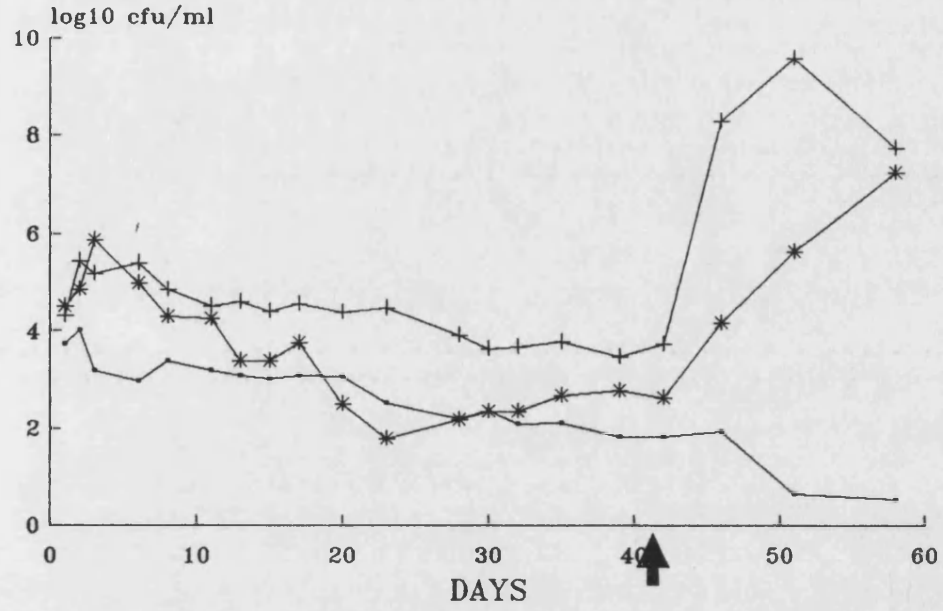


Figure 7 Persistence of *S.enteritidis* in albumen *in vitro*

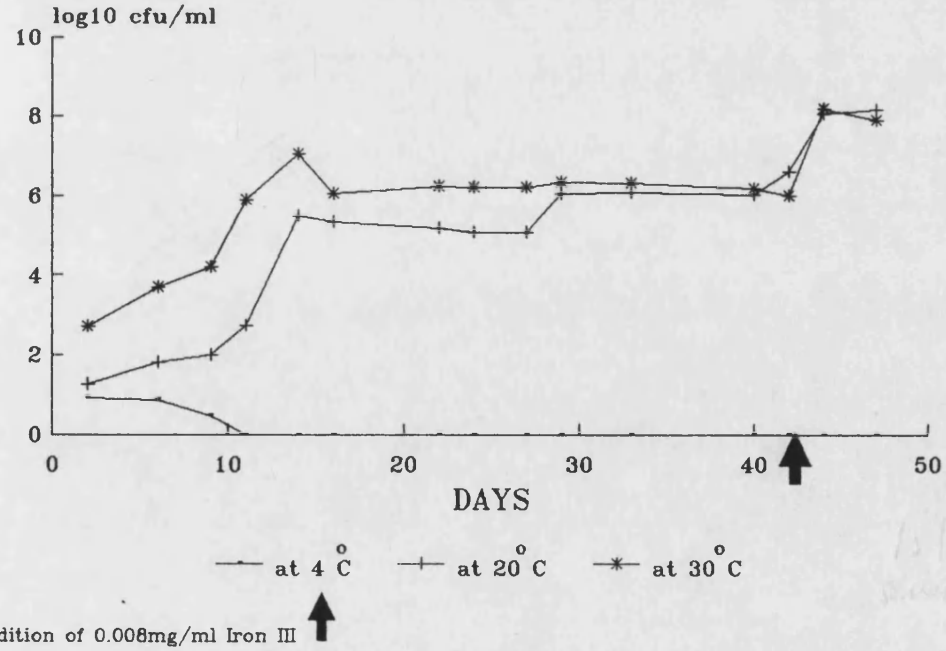


Figure 8 Persistence of *S.hadar* in albumen *in vitro*

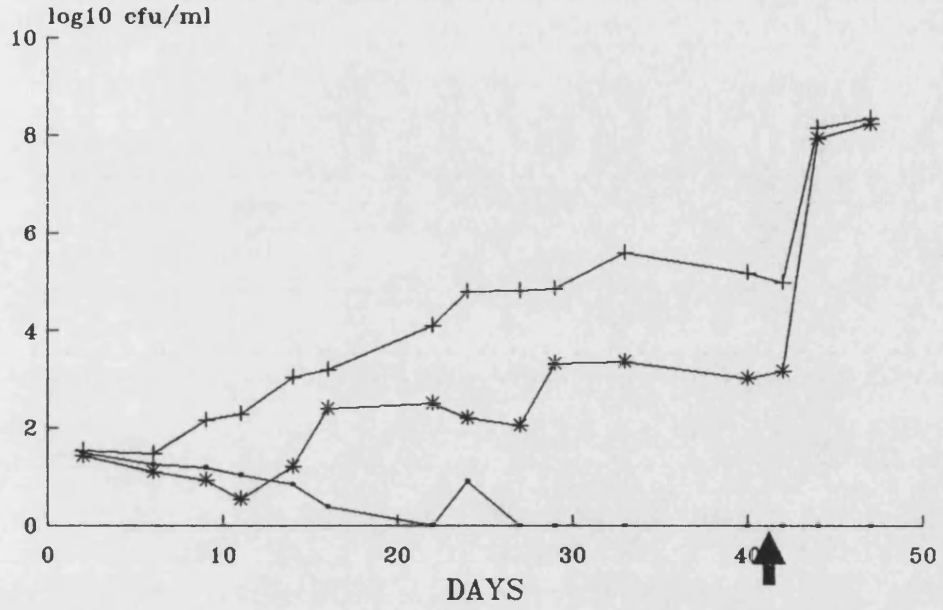


Figure 9 Persistence of *S.worthington* in albumen *in vitro*

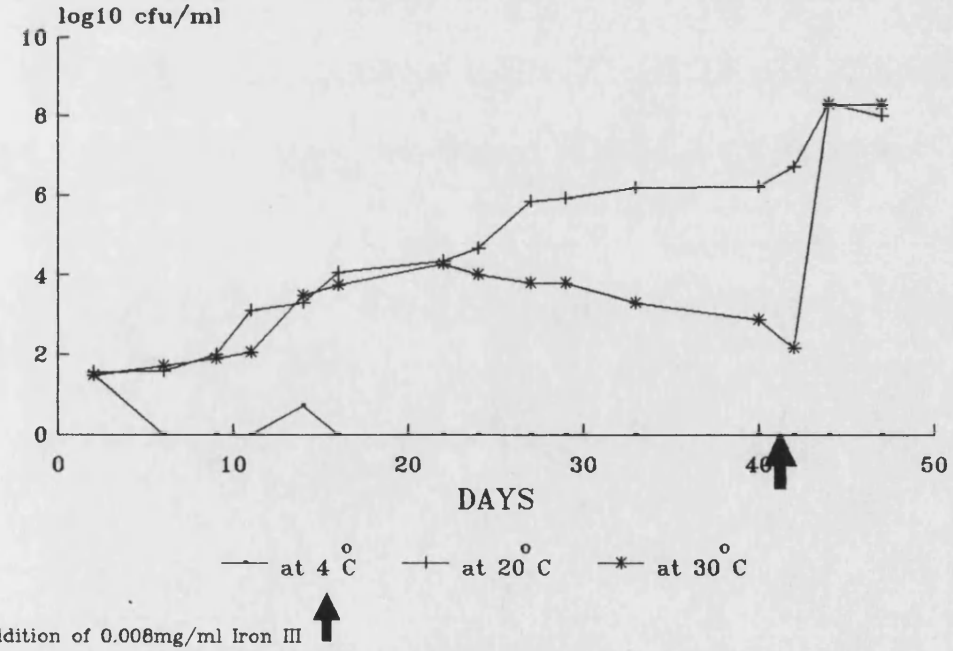


Figure 10 Persistence of *S.ohio* in albumen *in vitro*

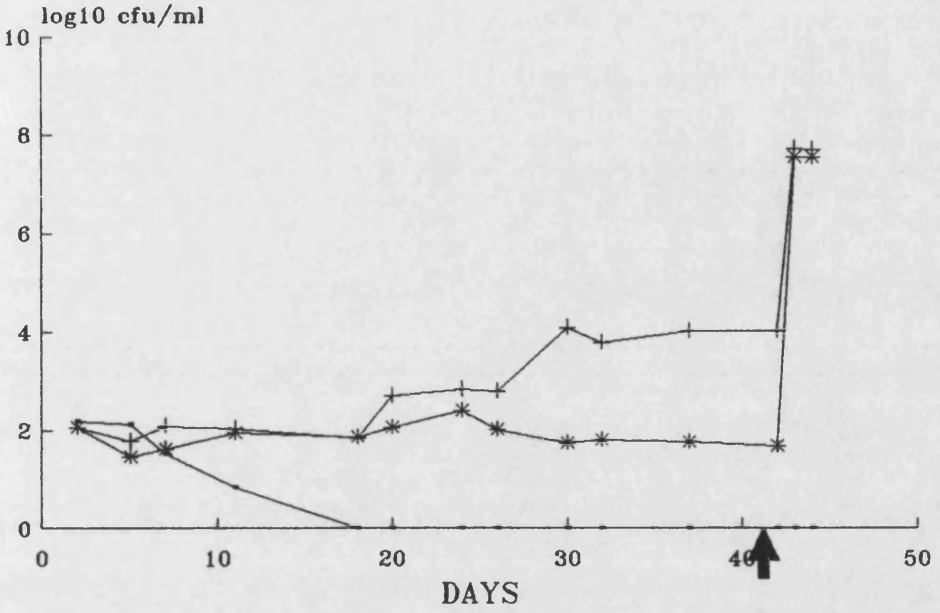


Figure 11 Persistence of *S.waycross* in albumen *in vitro*

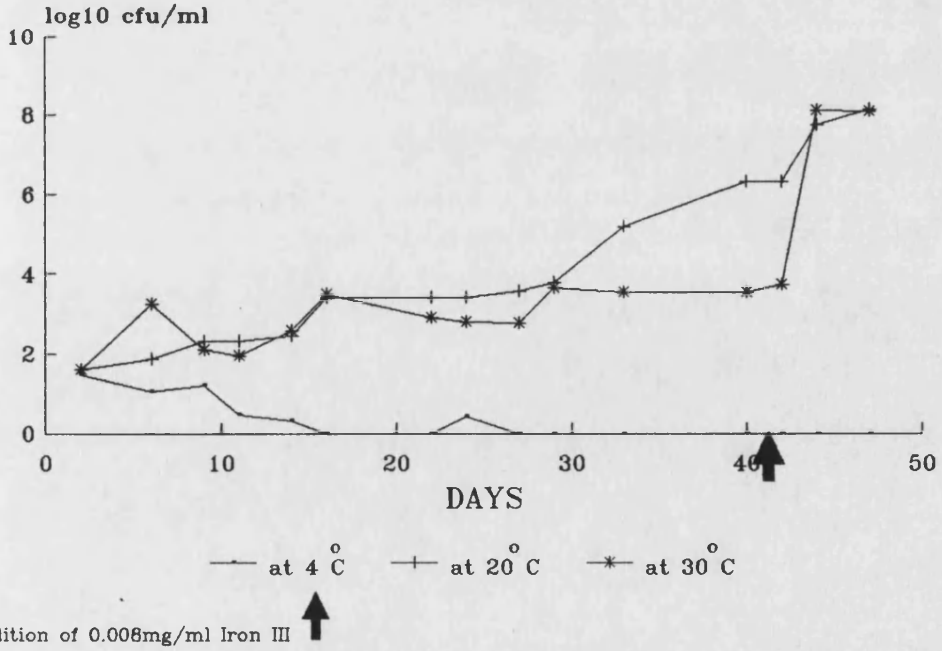
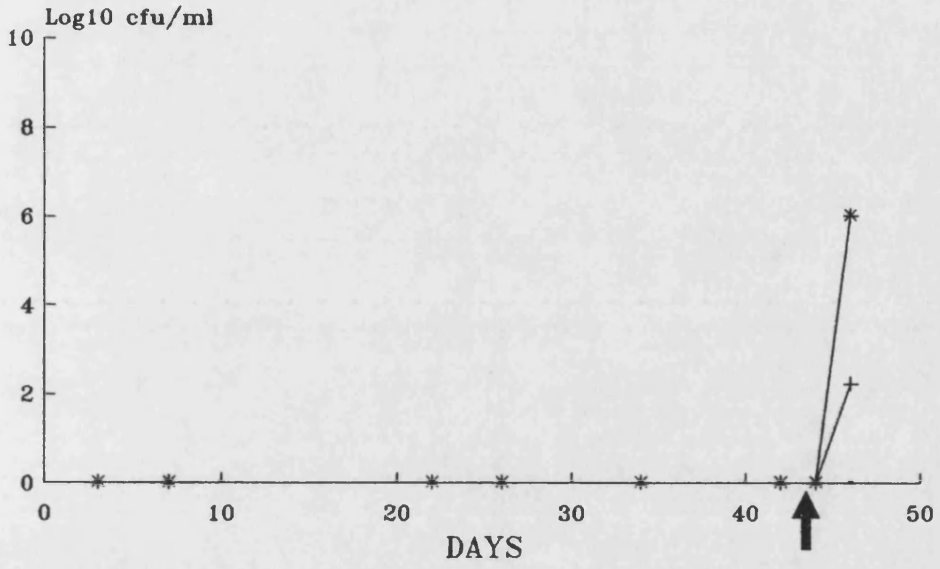
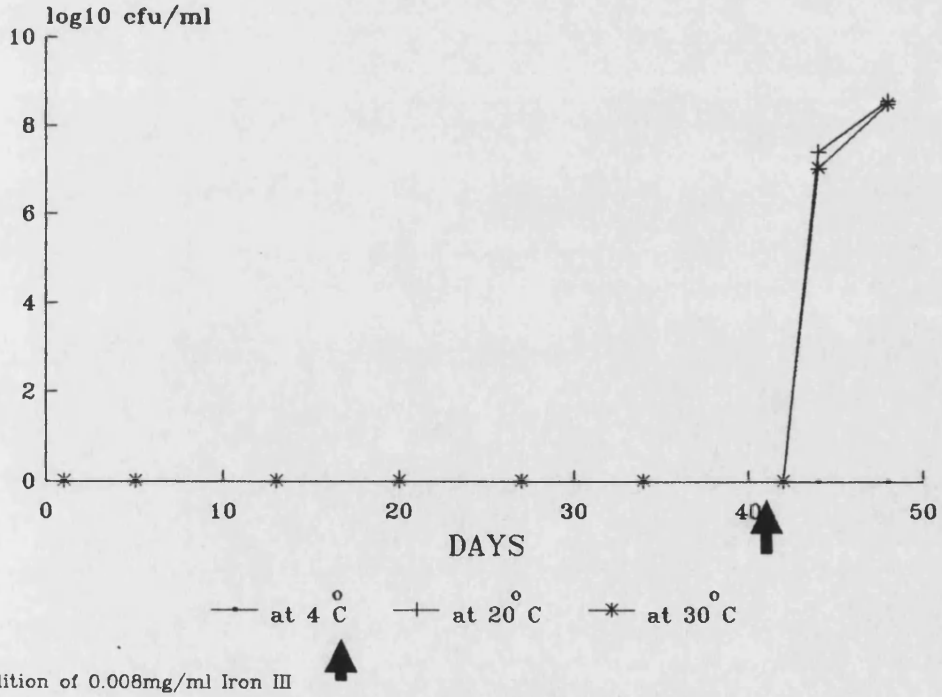


Figure 12 Persistence of different sizes of inocula of *S.enteritidis* in albumen *in vitro* (a) ca. 10 cfu/ml

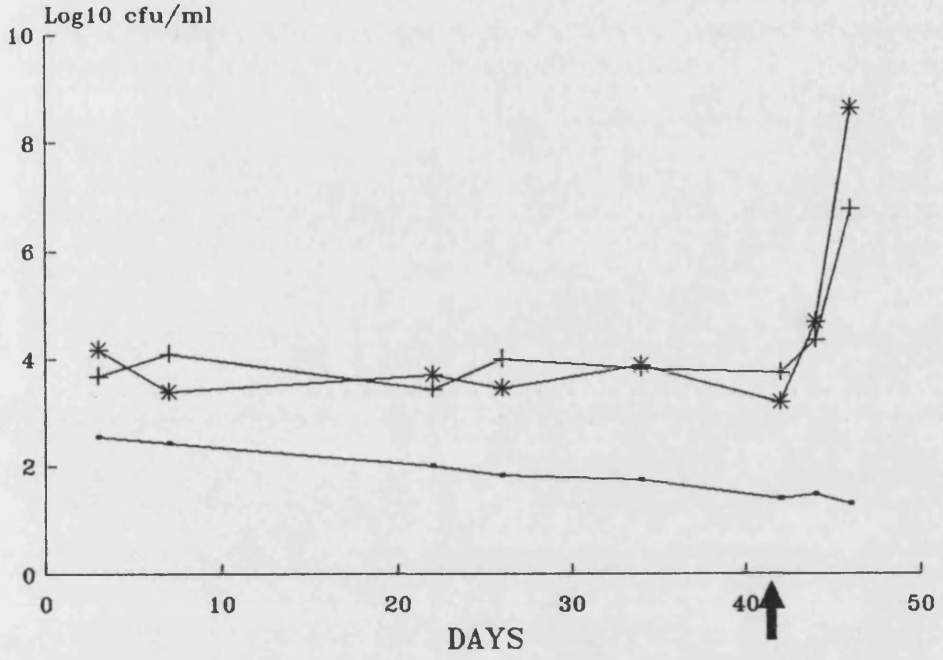


(b) ca. 100 cfu/ml



addition of 0.008mg/ml Iron III

(c) ca. 1000 cfu/ml



(d) ca. 1,000,000 cfu/ml

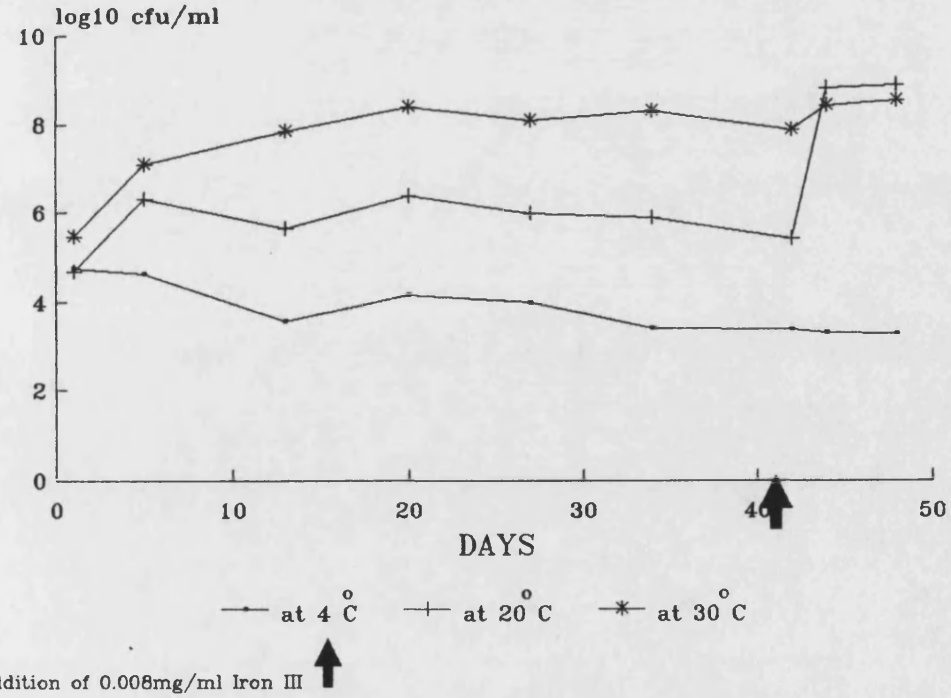
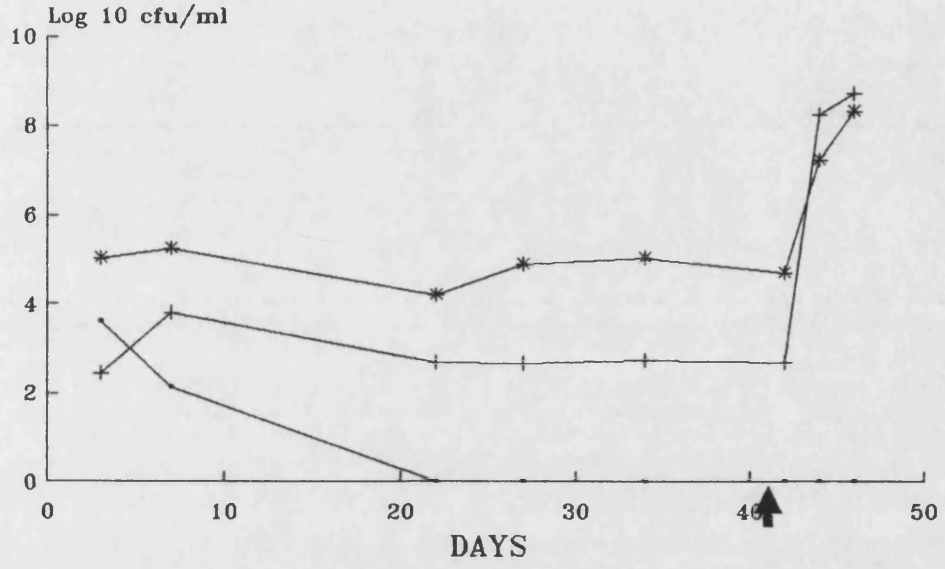


Figure 13 Persistence of different sizes of inocula of *S.hadar* in albumen *in vitro*

(a) ca. 1000 cfu/ml



(b) ca. 1,000,000 cfu/ml

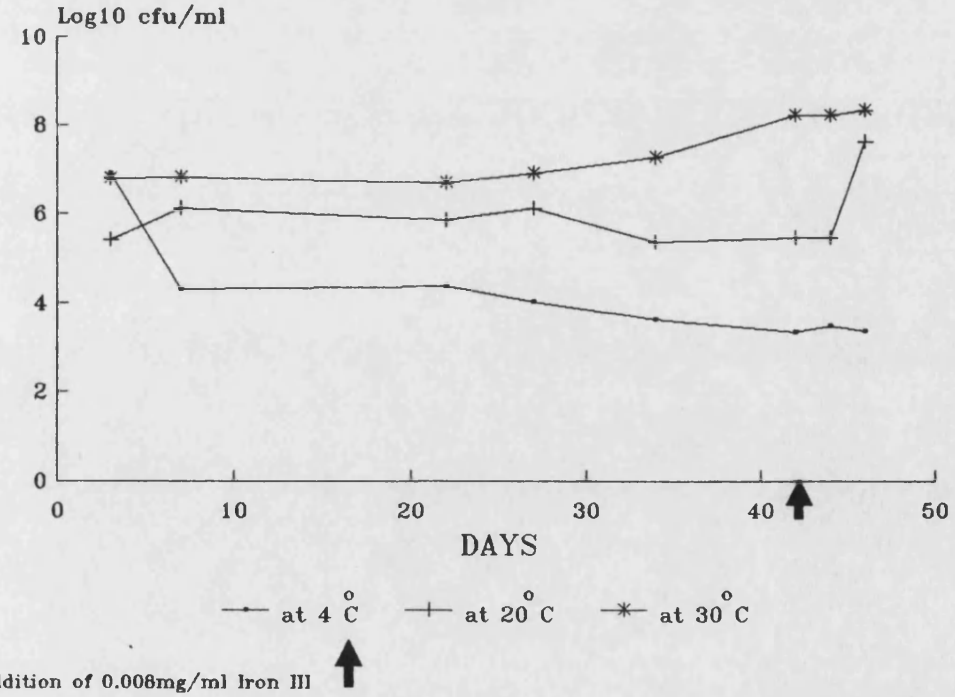
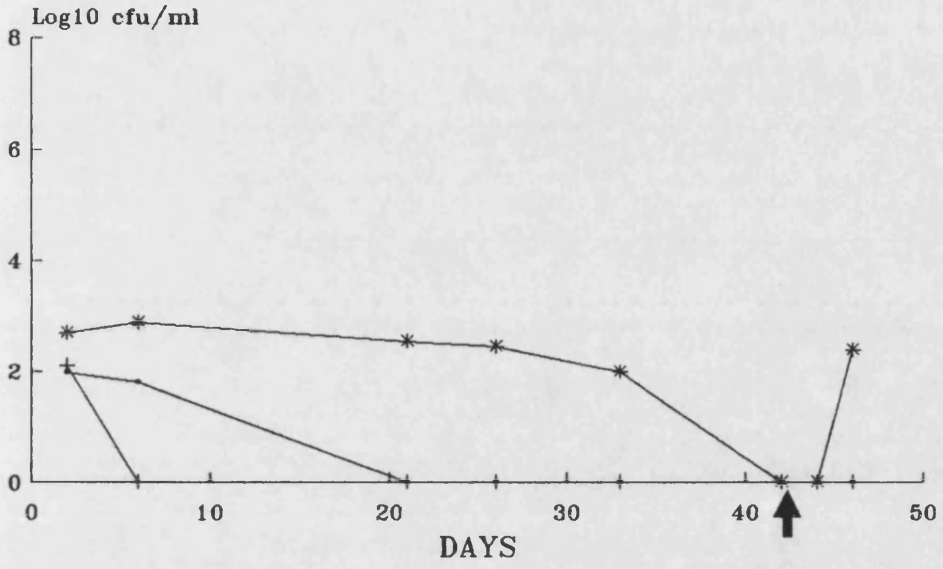


Figure 14 Persistence of different sizes of inocula of *S.pullorum* in albumen *in vitro* (a) ca. 1000 cfu/ml



(b) ca. 1,000,000 cfu/ml

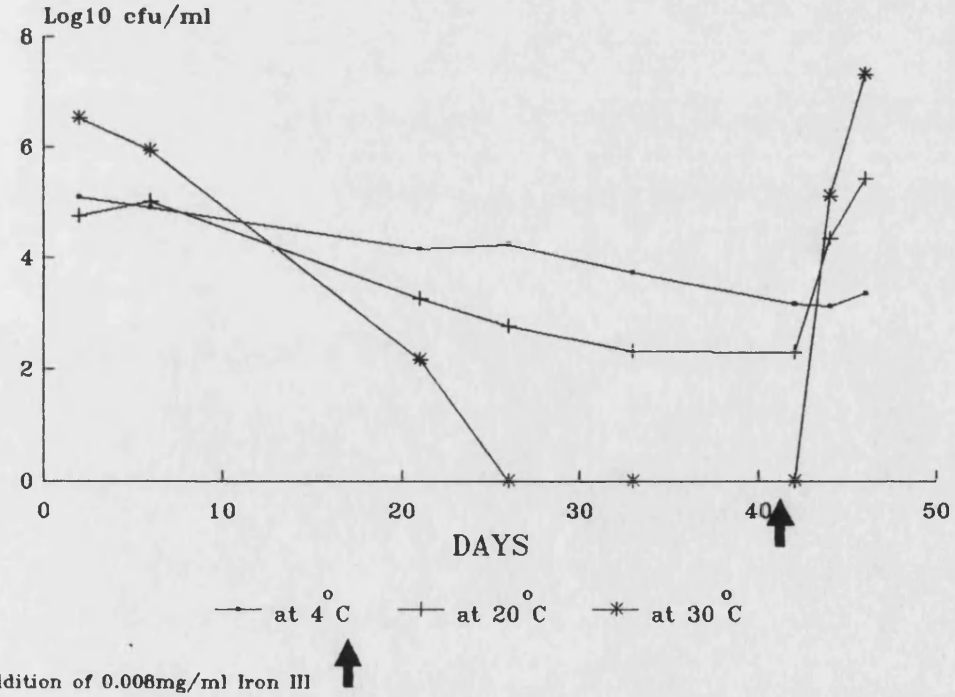


Table 11. The motility of *S.enteritidis* in albumen with nutritional supplements at 4 or 25 ^{0}C

Supplement	Number of days on which motility was observed at ^{0}C	
	4	25
NH_4Cl	2*	7-40
Growth factor solution(GF)	2	7-40
Faecal extract	5-21	7-50
Ferric ammonium citrate	30	>50
Control	2-20	7-50
Aged albumen	2-12	7-40

* The values were obtained from 3 experiments

A minimum of 200 cells examined (x1000 phase contrast) per sample. Observations were made daily until motility ceased.

NH_4Cl, 1mg/ml final concentration

Growth factor solution (GF), mg/ml, *p*-aminobenzoic acid, 10; folic acid, 1.0; Vitamin B_{12}, 1.0; nicotinic acid, 1.0; pantothenic acid, 1.0; thiamine, 1.0; biotin, 1.0; 1 ml added to albumen.

Faecal extract, 1/10 dilution, 1 ml added to albumen

Ferric ammonium citrate, 0.008mg/ml final concentration

Aged albumen, stored for 7 days at 30 ^{0}C before inoculation

Table 12. The percentage of motile cells of *S. enteritidis* in supplemented albumen after 5 days at 4 or 25 ^{0}C

Supplement	% of cells remaining motile at (^{0}C) 4	25
NH_4Cl	0-2.5*	55-72
Growth factor solution(GF)	0-5	30-37
Faecal extract	32-60	72-85
Ferric ammonium citrate	15-32	45-98
Control	0-25	27-55
Aged albumen	0-10	14-55

* A range of values were obtained from 3 experiments

A minimum of 200 cells examined (x1000 phase contrast) per sample

NH_4Cl, 1 mg/ml final concentration

Growth factor solution (GF), (mg/ml), *p*-aminobenzoic acid, 1.0; folic acid, 1.0; vitamin B_{12}, 1.0; nicotinic acid, 1.0; pantothenic acid, 1.0; thiamine, 1.0; riboflavin, 1.0; biotin, 1.0; 1 ml added to albumen

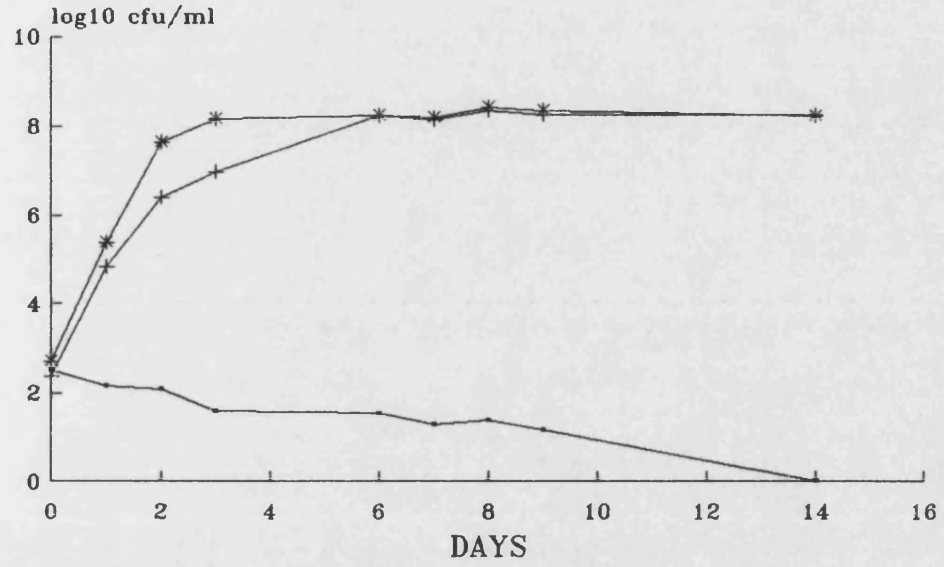
Faecal extract, 1/10 dilution, 1 ml added to albumen

Ferric ammonium citrate, 0.008 mg/ml final concentration

Aged albumen, stored for 7 days at 30 ^{0}C before inoculation

Figure 15 Behaviour of *S.enteritidis* in albumen *in vitro* supplemented with

(a) faecal extract



(b) iron-reduced faecal extract

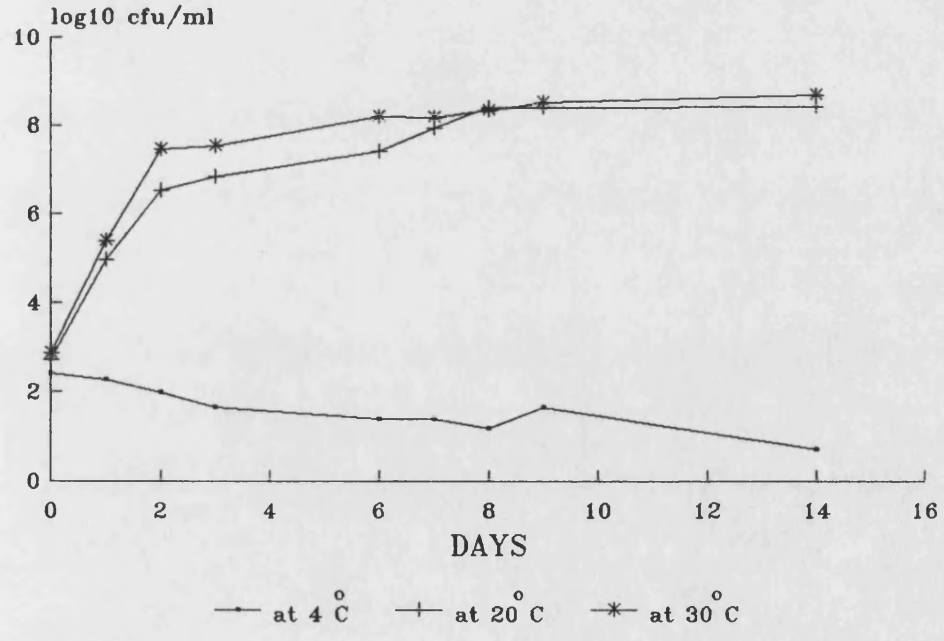
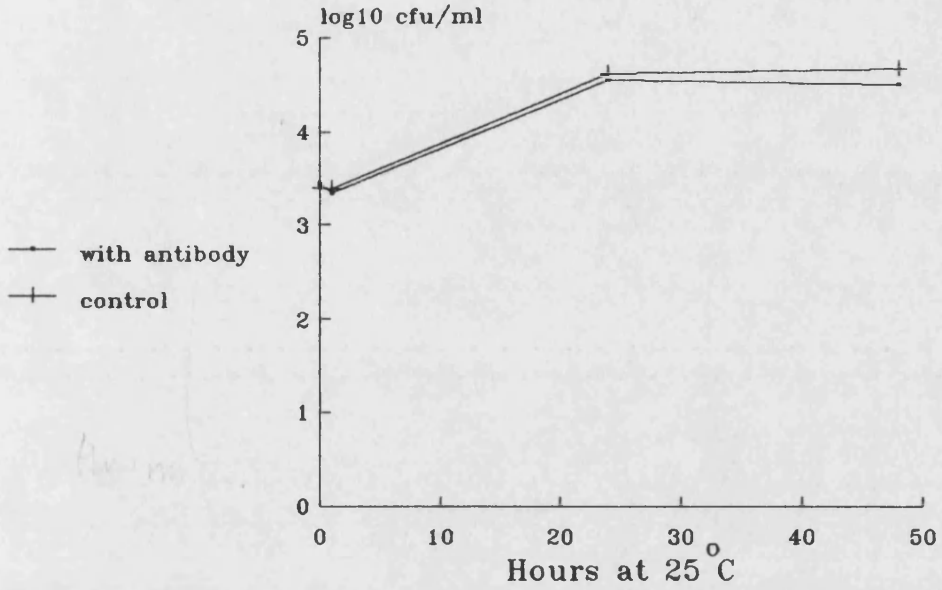
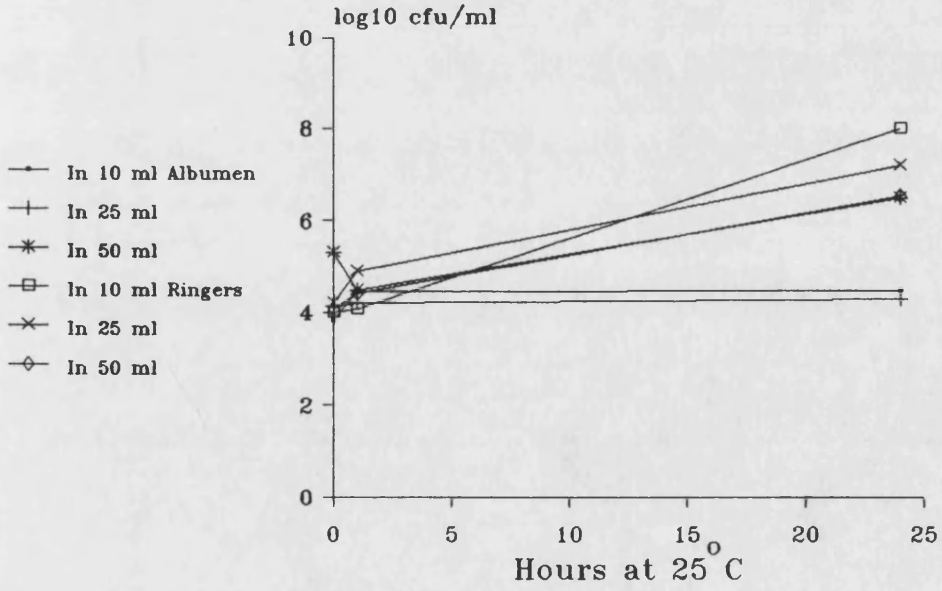


Figure 16 The fate of *S.enteritidis* in albumen with and without antibody



10,000 inoc, 0.15 ml antibody (titre 1/240)

Figure 17 The influence of antibody on *S.enteritidis* in albumen or Ringers



10,000 cfu/ml, 0.15 ml antibody (titre 1/240)

SECTION 2

A study of the ability of *S.enteritidis* to form somnicells

In the results presented in the persistence studies in Section 1, it was observed frequently that *S.enteritidis* failed to be detected in albumen for periods lasting upto 42 days (Figure 12a & b). After the addition of ferric ammonium citrate, however, the organisms were again culturable and detectable by the plate count method. In this section therefore I sought to answer the following questions. (1) Does the duration of stress determine the time required to resuscitate stressed cells ? (2) Can *Salmonella enteritidis* be induced to form somnicells in albumen ?

Previous studies including the present one of the infection process of eggs have used cultures that would be deemed physiologically fit. It is open to debate whether or not such a status obtains when eggs are infected by the oviducal, transovarian or trans-shell routes. It is not possible to define precisely all the factors that would impair the physiological fitness of organisms in nature. The literature does place emphasis, however, on the adverse effects of starvation. In the present study starvation and other forms of stress were achieved by several methods before salmonellas were inoculated into albumen *in vitro*. Two methods were adopted in attempts to induce the somnicell state. One involved the growth of *S.enteritidis* in nutrient broth overnight at 37 ^{0}C. The cells were then inoculated into Tris buffer (pH 7 or 9) and stored at 37 ^{0}C. After

varying lengths of time the cells were transferred into albumen stored at 37 ^{0}C. The second involved time/temperature variations in the treatment of the initial culture in nutrient broth. The cells were then transferred to Tris buffer (pH 9) and eventually inoculated into albumen and stored at 20 or 37 ^{0}C.

MATERIALS AND METHODS

(1) A study of the time required to resuscitate *S.enteritidis* cells in relation to the duration of the starvation period

An overnight (18h) culture of *S.enteritidis* was washed (x2) and resuspended in Tris dihydroxymethyl aminoethane (Tris buffer), pH 9 at 25 ^{0}C. On day one a sample was spun down (2000 g for 10 min), washed (x2) in saline (0.85%) and added to albumen. On day 2 a second sample was treated in the same manner. This process was repeated for 13 days. The ability of these cells to be resuscitated was monitored throughout the experiment by the presence or absence of growth in Buffered peptone water stored overnight or longer at 30 ^{0}C (g/l; peptone, 10; NaCl, 5.0; Na_2PO_4, 3.5; KH_2PO_4, 1.5).

(2) The persistence of stressed cells of *S.enteritidis* in albumen *in vitro*

Albumen was harvested as described previously. To 70 ml of albumen was added a cell suspension that had been treated as follows. The culture grown overnight in nutrient broth at 37 ^{0}C was washed in saline (x2) and transferred to

Tris buffer at pH 9 (similar to albumen) and stored at 20 ^{0}C for 3 days. It was then spun down (2000 g for 10 min), washed in saline and diluted such that 0.1 ml contained ca. 3 x 10^{4} cfu.

Duplicate samples were stored at 4, 20 or 30 ^{0}C and ferric ammonium citrate (final concentration 0.008 mg/ml Fe^{3+}) was added to the albumen on the 42^{nd} day of incubation. Viable counts were obtained on XLD in duplicate with incubation for 48 h at 37 ^{0}C.

A comparison of methods used to determine cell viability in Tris buffer and albumen

An overnight (18 h) *S.enteritidis* culture (10^{9} cfu/ml) in nutrient broth stored at 37 ^{0}C was washed in saline (x2) and inoculated into Tris buffer (pH 9) for 3 days storage at 20 ^{0}C. The culture was then washed (x2) in saline before transfer to Tris buffer (pH 9) and stored at 20 ^{0}C until day 16, at which time the cells were again washed (x2) in saline and finally inoculated into albumen. The number of viable cells in Tris buffer and albumen was determined by the following methods:

(a) Plate count. Viable counts were obtained by spreading 0.1 ml of an appropriate dilution on XLD or Nutrient agar (Lab M) with incubation for 48 h at 37 ^{0}C.

(b) Postgate's method (1961). Clean microscope slides (stored in alcohol) were flame dried. A plastic annulus (22 mm external diameter x 1.2 mm deep) was placed on the slide. A 0.24 ml portion of sterile nutrient agar was placed inside the annulus and stored in a Petri dish until ready for use.

A loopful of sample was spread on the surface of the agar and allowed to stand in the Petri dish for 10 min. A clean coverslip was placed over the annulus and a seal created by touching a loopful of distilled water to the interface of the annulus and coverslip. The slide was then stored at 37 ^{0}C for 2-4 h. It was then allowed to cool to room temperature before being inspected under phase contrast microscopy (x1000). Micro-colonies are scored as one viable unit and single cells as one dead unit. Sufficient fields were counted to include 300 "objects". Viability was scored as the percentage of viable to non-viable cells.

(c) Acridine Orange epifluorescent technique (Rollins and Colwell 1986). Samples (1 ml) were suspended to a final volume of 10 ml in 0.1M KH_2PO_4 (BDH: adjusted to pH 7.2 with NaOH). Diluted samples were filtered onto a 25 mm cellulose nitrate filter (black; 0.45 µm, Sartorius). Filters were then stained with 0.01% (w/v) Acridine Orange (Sigma) and examined by epifluoresence microscopy. Live cells stained orange and dead ones green (organic particles stained red). A blank was run also to determine the presence of any contaminating bacteria.

(d) Trypan Blue (Sigma- Anon 1991b). A cell suspension was prepared in Hanks balance salts solution (HBS - Appendix 1).

To a test tube was added 0.5 ml of trypan blue (0.4% in 0.8%, w/v, saline), 0.3 ml of HBS and 0.2 ml of diluted cell suspension. The solution was mixed thoroughly and allowed to stand for 5-15 min. The solution was then placed on a haemocytometer grid (Weber Scientific International Ltd) with coverslip in place - care was taken not to overfill the

chamber. Live cells remained colourless and dead ones took up the dye. Cells on the top and left of the middle line of the square of the grid were counted, those touching the middle bottom and right were not. A minimum of 200 cells were counted for each sample. Number of cells/ml = average count/square x (5×10^4) x dilution factor.

The validity of the technique was tested by autoclaving a cell suspension before staining with trypan blue. The number of live cells were determined.

(e) Methylene Blue (Vairo 1961; 200 mg/l, - BDH). Equal volumes of cell suspension and methylene blue were mixed prior to microscopic examination (x1000). Live cells remained colourless and dead ones appeared blue.

Cell size was monitored throughout these experiments. A minimum of 200 cells were measured using phase contrast microscopy (x1000; Olympus BH2 and an eye piece micrometer - Nuember), and mean cell length was calculated.

RESULTS

A study of methods to induce quiescence and the recovery of somnicells of *S.enteritidis* in albumen

In this section I sought to answer the following questions. (1) Does the duration of stress determine the time required to resuscitate somnicells ? (2) Can *Salmonella enteritidis* be induced to form somnicells in albumen ?

Salmonella enteritidis grown in nutrient broth was transferred to Tris buffer (pH 9) with storage for varying

lengths of time before inoculation into albumen (at 20 ^{0}C). Resuscitation was monitored by the presence or absence of growth in buffered peptone water after incubation at 30 ^{0}C. From Table 13 it is apparent that after 7 days in Tris buffer (pH 9), *S.enteritidis* was not resuscitated. By increasing the length of time in Tris buffer/albumen, it became increasingly difficult to resuscitate the cells ie. from <18 initially to 48 h. Eventually resuscitation did not occur. This experiment indicated that after 48 h incubation no further organisms were recovered from the resuscitation medium. This incubation time was therefore adopted in the following trials for all plate counts.

At the outset attention was directed at the role of media in the recovery of stressed cells. An overnight culture in nutrient broth at 37 ^{0}C was washed in saline (x2), transferred to Tris buffer (pH 9) and stored at 20 ^{0}C for 3 days. It was then harvested by centrifugation (2000 g for 10 min), washed in saline and diluted such that the inoculum (0.1 ml) contained ca. 3 x 10^{4} cfu/ml. It is evident from Figure 18 that the numbers of organisms recovered on NA were greater than those on XLD agar. This difference was accentuated with an increase in the period of incubation of stressed cells. Such differences did not obtain with physiologically fit organisms (Figure 18). In view of these results, NA was used in subsequent studies.

Salmonella enteritidis was subjected to the following conditions. After overnight incubation in nutrient broth at 37 ^{0}C, the culture was washed in saline (x2) transferred to Tris buffer (pH 9), stored for 3 days at 20 ^{0}C and, after

being washed on 2 further occasions, inoculated into albumen. The behaviour of organisms treated in this way was markedly different (Figure 19) from that noted in studies of "unstressed" cells (Figure 6 & 7). In the former case there was a progressive decline in the number of cells such that none was recovered by day 20 at 20 ^{0}C and day 28 at 30 ^{0}C. There appeared to be spontaneous "viability" recovery of some organisms in albumen between days 35-41 at 30 ^{0}C. Even so the addition of ferric ammonium citrate resulted in very large populations developing within 24 h in albumen incubated at 20 and 30 ^{0}C, but not at 4 ^{0}C.

The design of the experiments discussed above was such that there was no means of distinguishing between cell death and the possible formation of somnicells that failed to grow on NA. Even so these initial experiments led to the belief that stressed *S.enteritidis* may well have been induced to form somnicells during storage in albumen, and identified the need for direct means of testing viability.

Many microscopical methods have been developed to identify viable cells in a population. In the present study, 3 staining methods (Trypan blue, Acridine orange and Methylene blue), and Postgate's slide culture method were assessed. In all instances, microscopical counts of 200 cells and viable counts (on NA) on the same culture were compared.

It is evident from Figure 20 that Trypan blue and Methylene blue gave the highest viability scores in a comparison of staining methods. Methylene blue was not adopted for further work because of the limited literature

on its use in bacteriology. Trypan blue, on the other hand, has been used in studies with bacteria (Table 9) and was adopted. Dead cells take up the Trypan blue, viable cells do not (Anon 1991b). Thus, for example, more than 98% of the cells in an autoclaved culture of *S.enteritidis* stained blue whereas none of those in an incubated control did so. There was an excellent correlation also between the number of unstained (unstressed) cells and the viable count on NA when sampled in sequence (Table 14).

Two approaches were adopted to stress cells (Table 15). In trial 1 and 2, *S.enteritidis* was grown overnight in nutrient broth at 37 ^{0}C before the harvested (2000 g x 10 min) population was stressed by storage in Tris buffer (pH 9) for varying lengths of time. The cells were transferred eventually to albumen. In trials 3 and 4 various time/temperature stresses were applied to a growing organism. Tris buffer was used in all experiments. It was presumed that stress was the outcome of an interplay of nutrient deprivation, ammendment of cell walls (removal of divalent cations) and unfavourable pH.

Trial 1

Salmonella enteritidis grown overnight in nutrient broth at 37 ^{0}C was washed and stored in Tris buffer (pH 9) before transfer to albumen on days 2 (Figure 21), 6 (Figure 22) and 14 (Figure 23). In these trials the storage temperature of 37 ^{0}C was chosen, as it provides the optimum incubation temperature for *S.enteritidis*. In each case there was a common trend namely an apparent loss of viability of the organism in albumen or Tris buffer as

indexed by the plate count method but not with Trypan blue (direct) count. The addition of ferric ammonium citrate to the albumen containing organisms transferred from Tris buffer on day 2 led to the plate count equalling the direct counts probably within 24 hours. This was the situation also with those organisms inoculated into albumen on days 6 and 14.

Trial 2

The experiments described above provided possible evidence that stressed *S.enteritidis* formed somnicells when incubated in albumen or Tris buffer. Additional studies were done to determine whether or not somnicells formed in Tris buffer adjusted to pH 7 and incubated at 37 ^{0}C. In this instance there was no demonstrable difference between total and viable counts when the organism was transferred from Tris buffer (pH 7) on the 2nd day of storage (Figure 24) or after repeated transfer from Tris buffer (pH 7) to Tris buffer (pH 7) on days 2, 4 and 6 and finally into albumen on day 14 (Figure 25). In practice there was slight growth of the organism in all instances. The results obtained from this subculturing regime but with Tris buffer adjusted to pH 9 were comparable to those obtained in Trial 1. Thus after transfer to albumen on day 2 (Figure 26), there was a complete loss of viability as judged by the plate count method but not by the staining method. On addition of ferric ammonium citrate, however, the numbers of viable organisms obtained by the plate count method equalled those of the direct count. There was, however, complete loss of viability, as judged by the plate count but not the staining

method, when *S.enteritidis* was transferred to albumen on day 14. Moreover the addition of ferric ammonium citrate to albumen did not result in the recovery of cell viability (Figure 27). It was assumed that the stresses imposed on the organism were too great for the transition from the somnicell state to culturable cells to occur with ferric ammonium citrate alone.

Trial 3

From these experiments it is evident that *S.enteritidis* grown overnight in nutrient broth at 37 ^{0}C was capable of retaining viability in Tris buffer or albumen. In other experiments *S.enteritidis* was stored initially in nutrient broth for 9 days at 4 ^{0}C before further incubation overnight at 37 ^{0}C. The cultures were then stored at 4 ^{0}C for 19 days, washed in saline (x2) before being inoculated into albumen or Tris buffer (pH 9) and incubated at 37 ^{0}C. As judged by the plate count method, organisms inoculated into albumen (Figure 28) showed a very slow decrease in viable numbers with time. On the addition of ferric ammonium citrate, the numbers of viable organisms in albumen detected by the plate count method equalled those scored by the direct count method. When cells were incubated in Tris buffer for 31 days before transfer to albumen, the numbers recovered on NA declined rapidly. Viable organisms were not detected by this method until the addition of ferric ammonium citrate to albumen. Again this led to the plate counts equalling the direct counts (Figure 29).

Trial 4

Incubation of the organisms at stress temperatures (37 ^{0}C overnight, 24 h at 44 ^{0}C and 24 h at 37 ^{0}C) led to the number of organisms recovered from albumen stored at 20 ^{0}C (Figure 30) on NA declining progressively to below detectable levels over 22 days. The addition of ferric ammonium citrate on the 29th day led to the plate counts equalling the direct counts. In this trial the storage temperature (20 ^{0}C) was chosen for comparison with the previous trials (37 ^{0}C). An attempt was made to investigate the possibility that a lower storage temperature may increase the ability of *S.enteritidis* to recover from the somnicell state. Comparable results were obtained also when *S.enteritidis* was transferred after 31 days of storage from Tris buffer (pH 9) to albumen (Figure 31). Again the addition of ferric ammonium citrate led to the recovery of culturability such that the plate counts equalled the direct counts.

Throughout these experiments cell size was measured but fractionation, considered to be a general reaction to stress (Koch 1979), was not seen. Infact a small number of cells (<10% of the population) were 5-6 µm long.

The results presented in the first 2 sections of this study indicate that organisms present in the albumen remain quiescent until the antimicrobial properties of the albumen are negated. Some of these cells may well enter the somnicell state. They are viable as determined by the direct count method, but non-viable by the plate count method. These cells may be induced to express their true

population size ie. become culturable or even multiply, in resuscitation media such as Buffered peptone water. This may be induced also by the addition of compounds which negate the antimicrobial properties of the albumen eg. ferric ammonium citrate. Resuscitation was only possible if the stresses on the organism had not been too great to prevent recovery and if the organism was stored at growth promoting temperatures. It was noteworthy that *S.enteritidis* did not appear to undergo fractionation when exposed to stress.

Table 14. The resuscitation of *S.enteritidis* in Buffered peptone water at 30 ^{0}C after storage in Tris pH 9 at 37 ^{0}C

No.days in Tris pH 9	No.days in Albumen	Resuscitation achieved after (days)	Resuscitation time taken in BPW (h)
0	30	30	<18
1	29	30	<18
2	28	30	<18
4	26	5	24
5	25	8	24
6	24	7	48
7	23	13	48
8	22	-	-
12	18	-	-
13	17	-	-

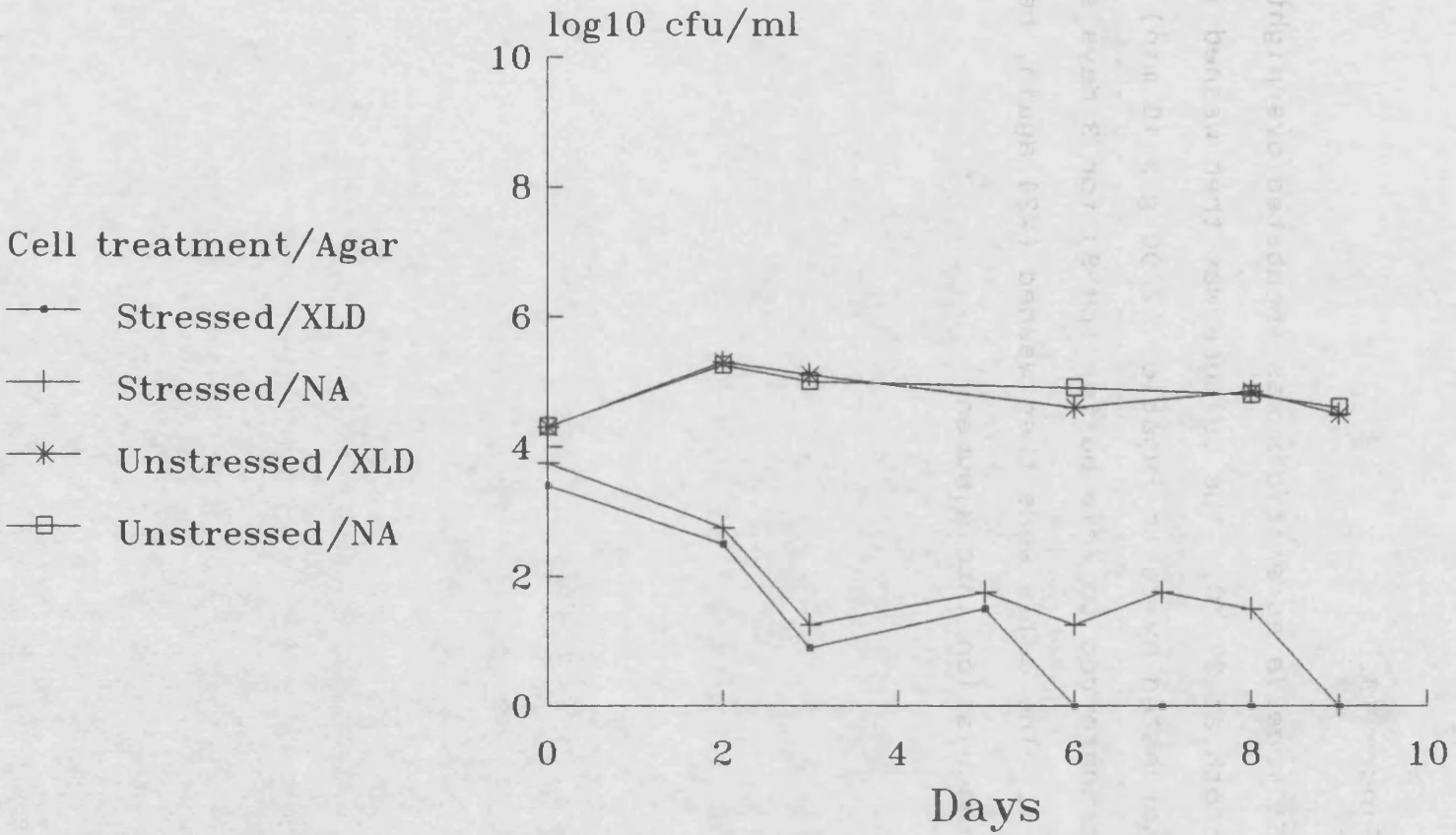
- resuscitation not achieved

This experiment was done twice, in duplicate on both occasions. The results show the average time required to resuscitated the organism. There was no variation in the number of days after which resuscitation was impossible. BPW - Buffered peptone water.

Figure 18.

Salmonella enteritidis was incubated overnight in nutrient broth at 37 ^{0}C. The culture was then washed in saline (x2), harvested by centrifugation (2000 g x 10 min) and transferred to Tris buffer (pH 9) for 3 days storage at 20 ^{0}C. The cells were then washed (x2) again, before inoculation into albumen.

Figure 18 The behaviour of stressed and unstressed *S.enteritidis* in albumen *in vitro* at 20 °C



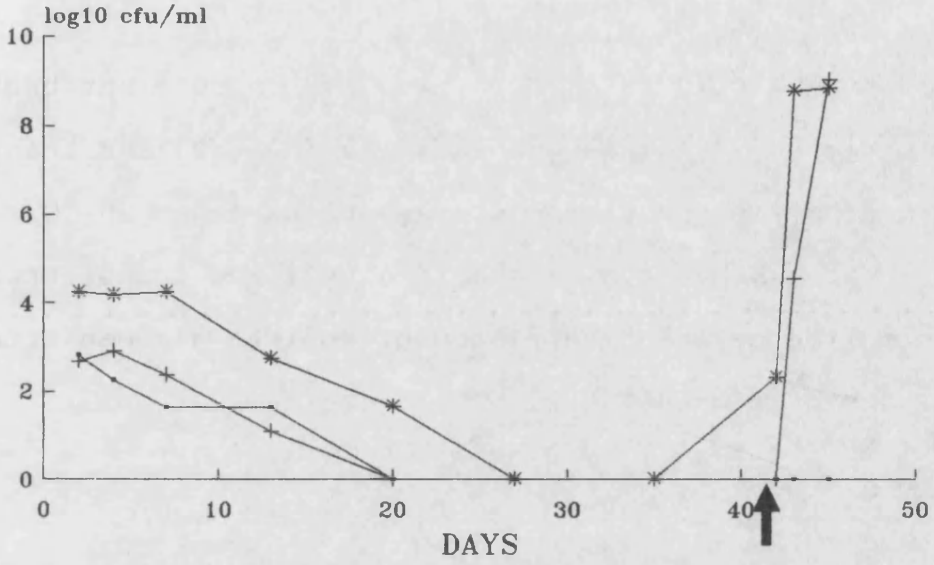
XLD and Nutrient Agar (NA) * = the results of 2 separate experiments

Figure 19.

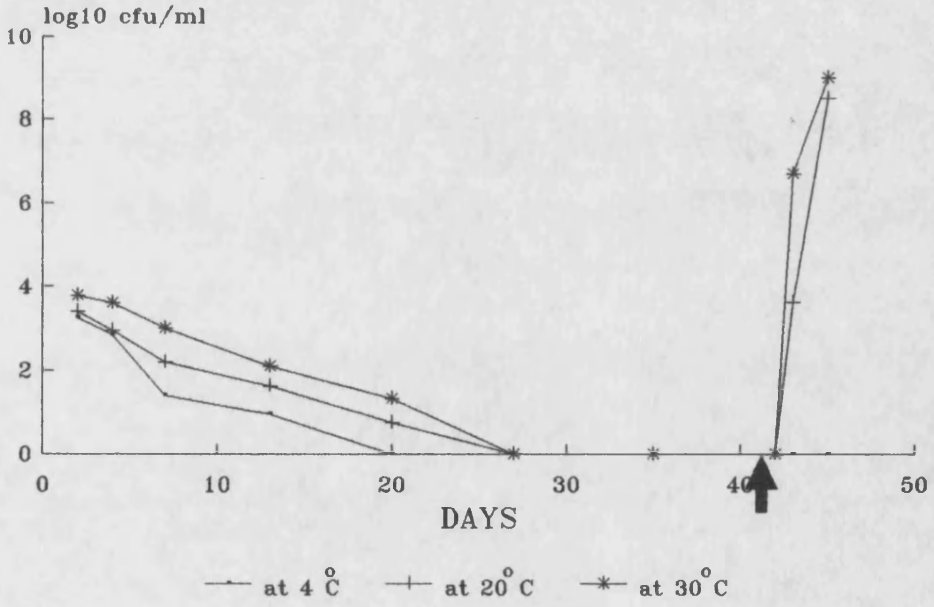
Salmonella enteritidis was incubated overnight in nutrient broth at 37 ^{0}C. The culture was then washed in saline (x2), harvested by centrifugation (2000 g x 10 min) and transferred to Tris buffer (pH 9) for 3 days storage at 20 ^{0}C. The cells were then washed (x2) again, before inoculation into albumen.

Figure 19 Fate of stressed *S.enteritidis* in albumen *in vitro*

(a) Experiment 1



(b) Experiment 2

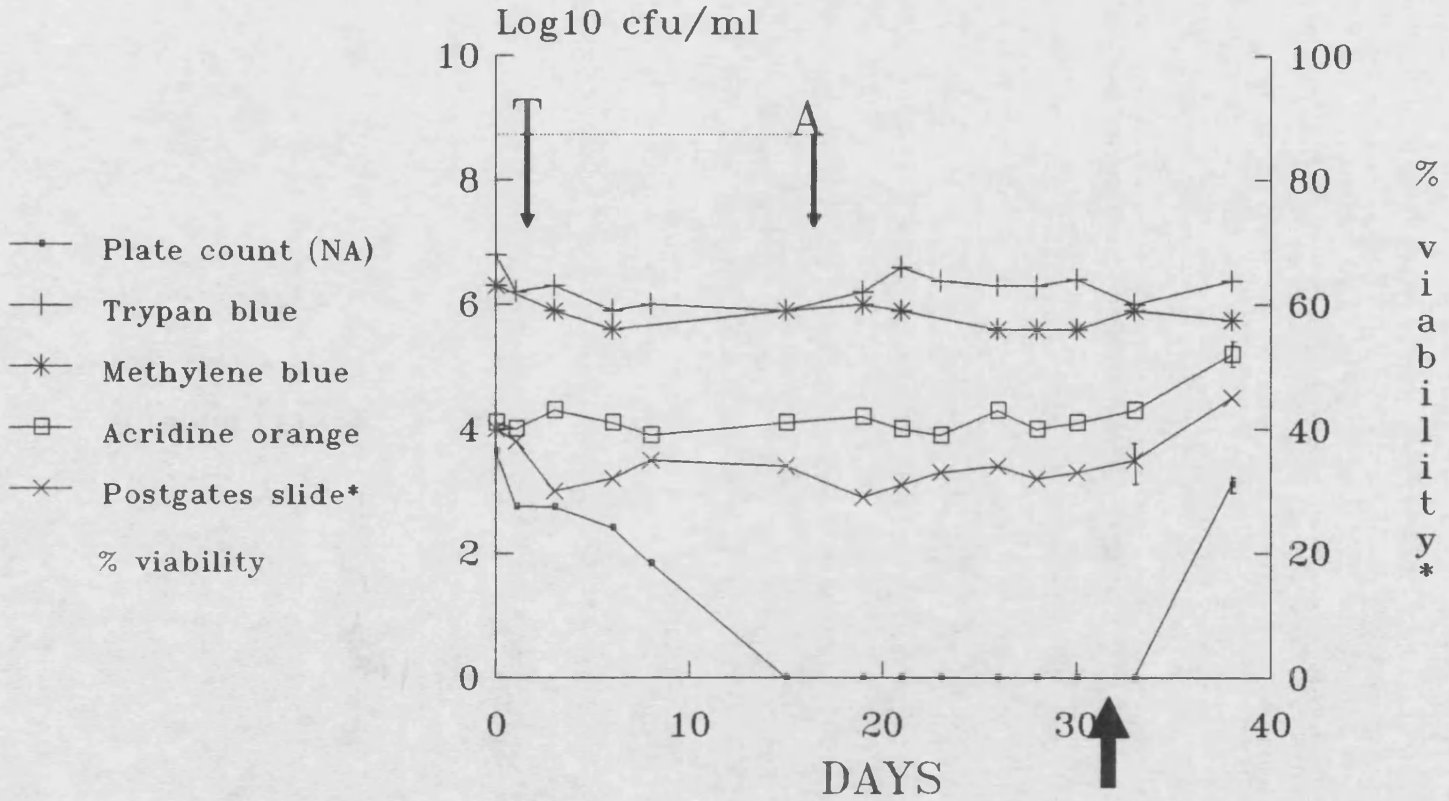


addition of 0.008mg/ml Iron III.
Results obtained on Nutrient Agar

Figure 20.

An overnight culture of *S.enteritidis* in nutrient broth incubated at 37 ^{0}C was washed in saline (x2) and transferred to Tris buffer (pH 9) for storage (2 days) at 20 ^{0}C. The cells were washed again in saline (x2) and transferred to Tris buffer before final inoculation into albumen after the 16th day of storage at 20 ^{0}C.

Figure 20 The behaviour of *S.enteritidis* in Tris buffer (pH 9) and albumen as indicated by plate counts and direct counts



T =Transfer to Tris, A = Transfer to albumen. Addition of 0.008mg/ml Iron III
Average of 2 experiments

Table 13. A comparison of the plate count method (NA) and direct count method (Trypan blue) on unstressed *S.enteritidis* cells from nutrient broth at 37 ^{0}C

Method	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
NA (cfu/ml)	3.2×10^{5}	4.4×10^{5}	2.7×10^{5}	5.6×10^{6}	7.2×10^{8}
Trypan blue (viable cell No.)	3.7×10^{5}	4.6×10^{5}	2.5×10^{5}	6.1×10^{5}	7.0×10^{8}

Salmonella enteritids was grown in nutrient broth overnight at 37 ^{0}C and diluted in Ringers solution. The number of cells present was determined by the plate count (duplicate plates) and direct count methods (a minimum of 200 cells). All results were obtained from the same culture sequentially.

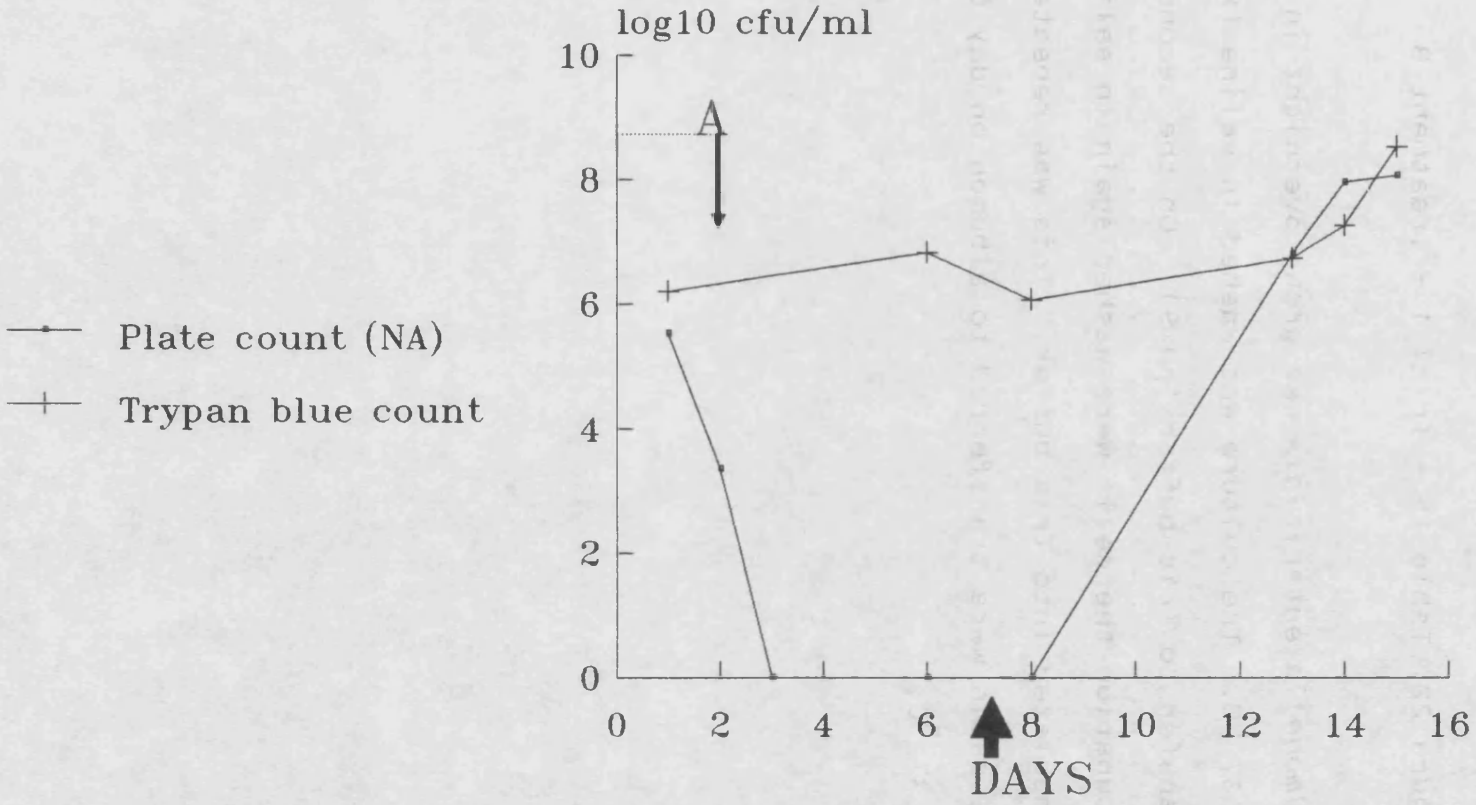
Table 14. Experimental conditions used to induce *S.enteritidis* to form somnicells in Tris buffer and albumen

Trial	Preparation of mother culture in nutrient broth	Transfer to *f*(^{0}C)		Transfer on day, to 2	4	6	14
1	overnight 37 ^{0}C	Tris pH 9 (37)	A	Albumen	-	-	-
	"	"	B	Tris	Tris	Albumen	-
	"	"	C	Tris	Tris	Tris	Albumen
2	overnight 37 ^{0}C	Tris pH 7* (37)	A	Albumen	-	-	-
	"	"	C	Tris*	Tris*	Tris*	Albumen
	overnight 37 ^{0}C	Tris pH 9 (37)	A	Albumen	-	-	-
	"	"	C	Tris	Tris	Tris	Albumen
3	9 days at 4^{0}C overnight at 37 ^{0}C, 19 days at 4 ^{0}C	Albumen (37)	D	-	-	-	-
	"	Tris pH 9 (37)	E	added to albumen on day 31			
4	Overnight at 37 ^{0}C, 24 h at 44 ^{0}C then 24 h at 37 ^{0}C	Albumen (20)	D	-	-	-	-
	"	Tris pH 9 (20)	E	added to albumen on day 31			

f = The culture was centrifuged (2000 g x 10 min), washed in saline (x2) before tranfer.
* = Tris buffer pH 7
- = No further treatment

A = Cells inoculated into albumen on day 2 at 37 ^{0}C
B = Cells transferred from Tris buffer to Tris buffer on day 2 and again on day 4, and finally into albumen on day 6. Stored at 37 ^{0}C
C = As B, but on day 6 transfer was from Tris to Tris before transfer to albumen on day 14. Stored at 37 ^{0}C
D = Cells transferred directly into albumen (day 0)
E = Cells transferred to albumen after 31 days in Tris

Figure 21 The behaviour of *S.enteritidis* in albumen at 37°C following stress in Tris buffer (pH 9)

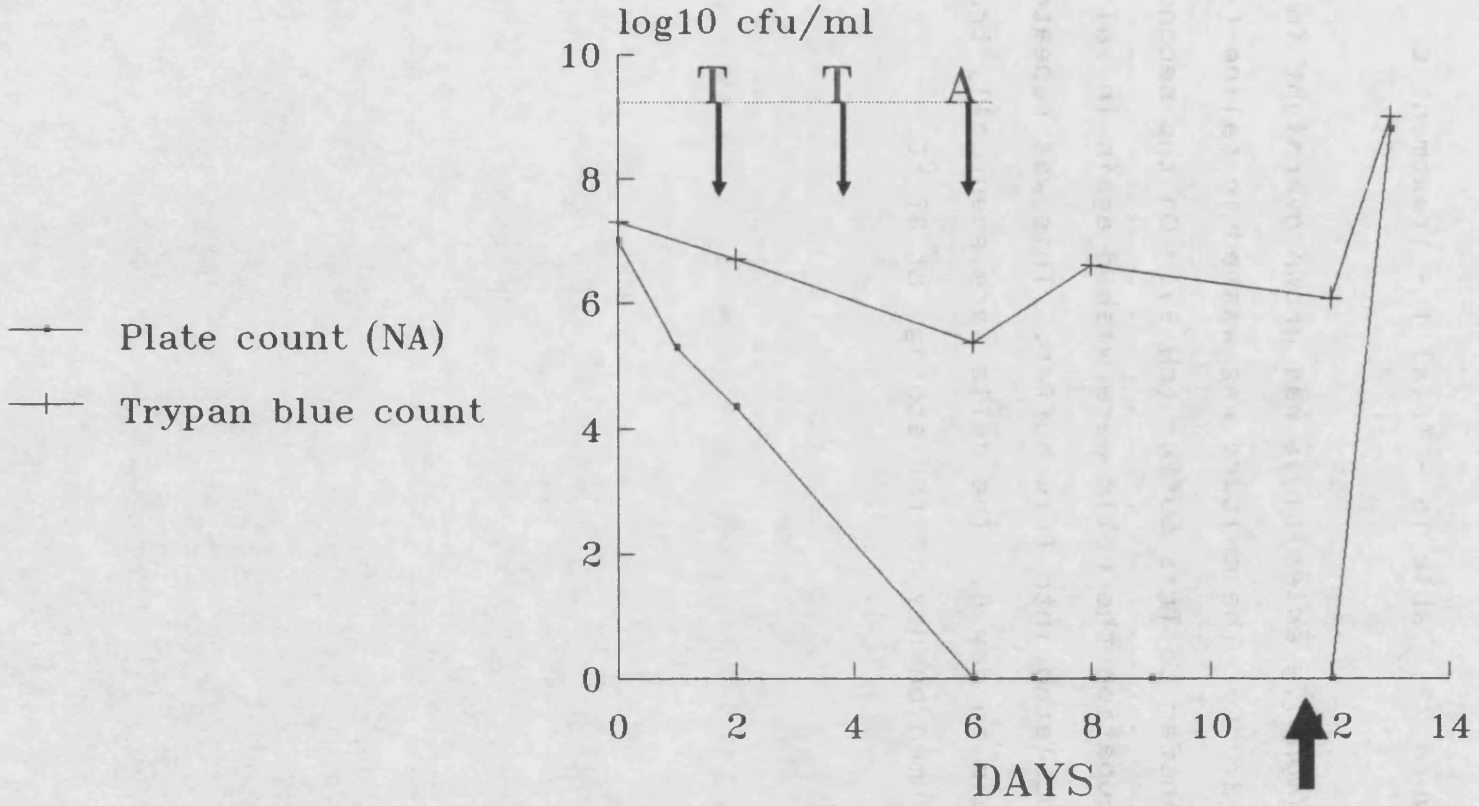


A = Transfer to Albumen.
Addition of 0.008mg/ml Iron III
Results of 1 experiment

Figure 22. Table 15 - Trial 1 - Treatment B

Salmonella enteritidis was grown overnight in nutrient broth at 37 ^{0}C. The culture was washed in saline (x2) before transfer to Tris buffer (pH 9). On the second day of incubation the cells were washed again in saline (x2) and inoculated into Tris buffer. This was repeated on day 4. The cells were transferred to albumen on day 6 for storage at 37 ^{0}C.

Figure 22 The behaviour of *S.enteritidis* in albumen at 37°C following stress in Tris buffer (pH 9)

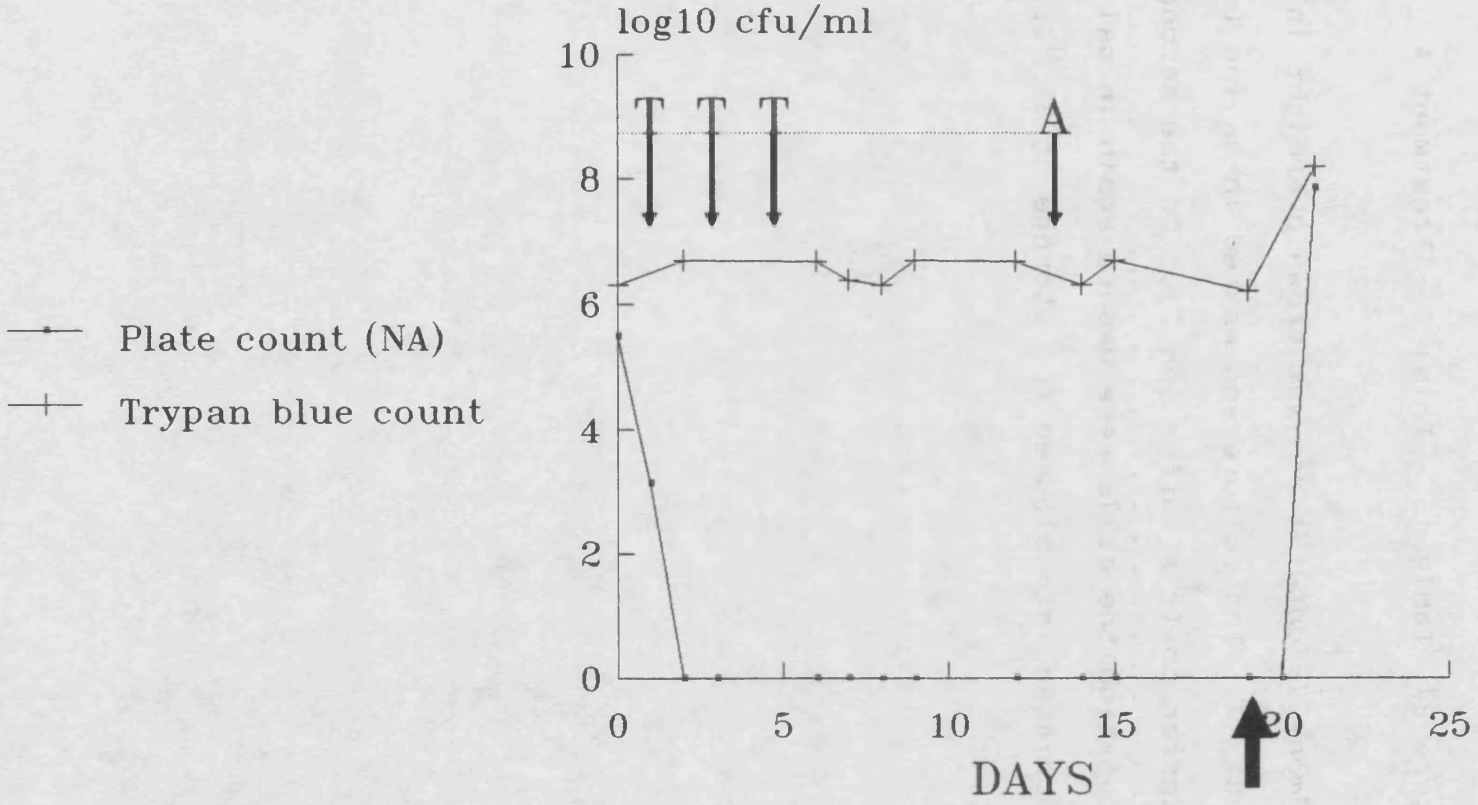


T = Transfer to Tris, A = Transfer to albumen. Addition of 0.008mg/ml Iron III
Results of 1 experiment

Figure 23. Table 15 - Trial 1 - Treatment C

Salmonella enteritidis was grown overnight in nutrient broth at 37 ^{0}C. The culture was washed in saline (x2) before transfer to Tris buffer (pH 9). On the second day of incubation the cells were washed again in saline (x2) and inoculated into Tris buffer. This was repeated on day 4 and again on day 6. The cells were eventually transferred to albumen on day 14 for storage at 37 ^{0}C.

Figure 23 The behaviour of *S.enteritidis* in albumen at 37°C following stress in Tris buffer (pH 9)

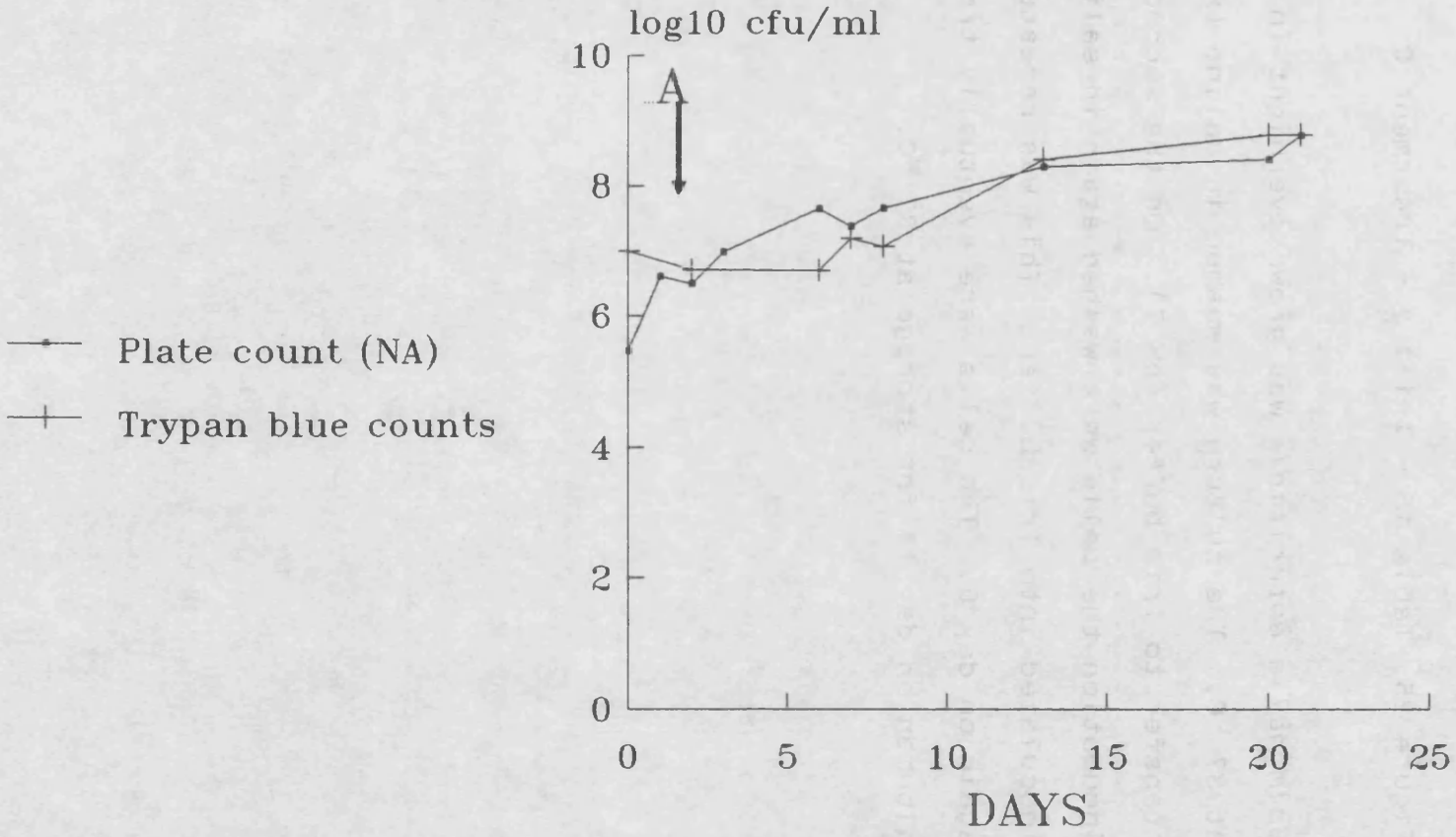


T = Transfer to Tris, A = Transfer to albumen. Addition of 0.008mg/ml Iron III
Result of 1 experiment

Figure 24. Table 15 - Trial 2 - Treatment A

Salmonella enteritidis was grown overnight in nutrient broth at 37 ^{0}C. The culture was washed in saline (x2) before transfer to Tris buffer (pH 7). On the second day of incubation the cells were washed again in saline (x2) and inoculated into albumen for storage at 37 ^{0}C.

Figure 24 The behaviour of *S.enteritidis* in albumen at 37°C following stress in Tris buffer (pH 7)

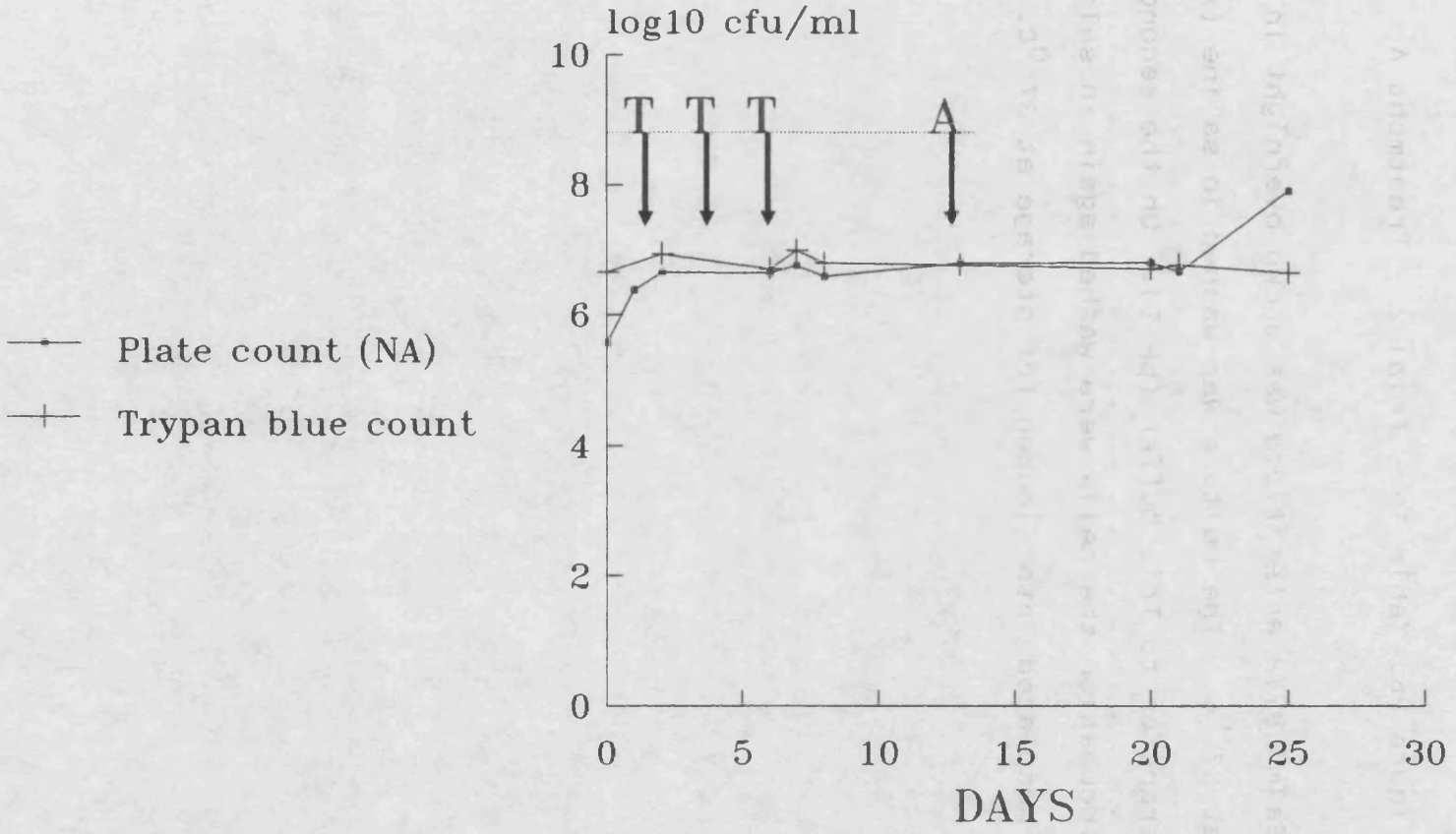


A = Transfer to albumen
Results of 1 experiment

Figure 25. Table 15 - Trial 2 - Treatment C

Salmonella enteritidis was grown overnight in nutrient broth at 37 ^{0}C. The culture was washed in saline (x2) before transfer to Tris buffer (pH 7). On the second day of incubation the cells were washed again in saline (x2) and inoculated into Tris buffer. This was repeated on day 4 and again on day 6. The cells were eventually transferred to albumen on day 14 for storage at 37 ^{0}C.

Figure 25 The behaviour of *S.enteritidis* in albumen at 37°C following stress in Tris buffer (pH 7)

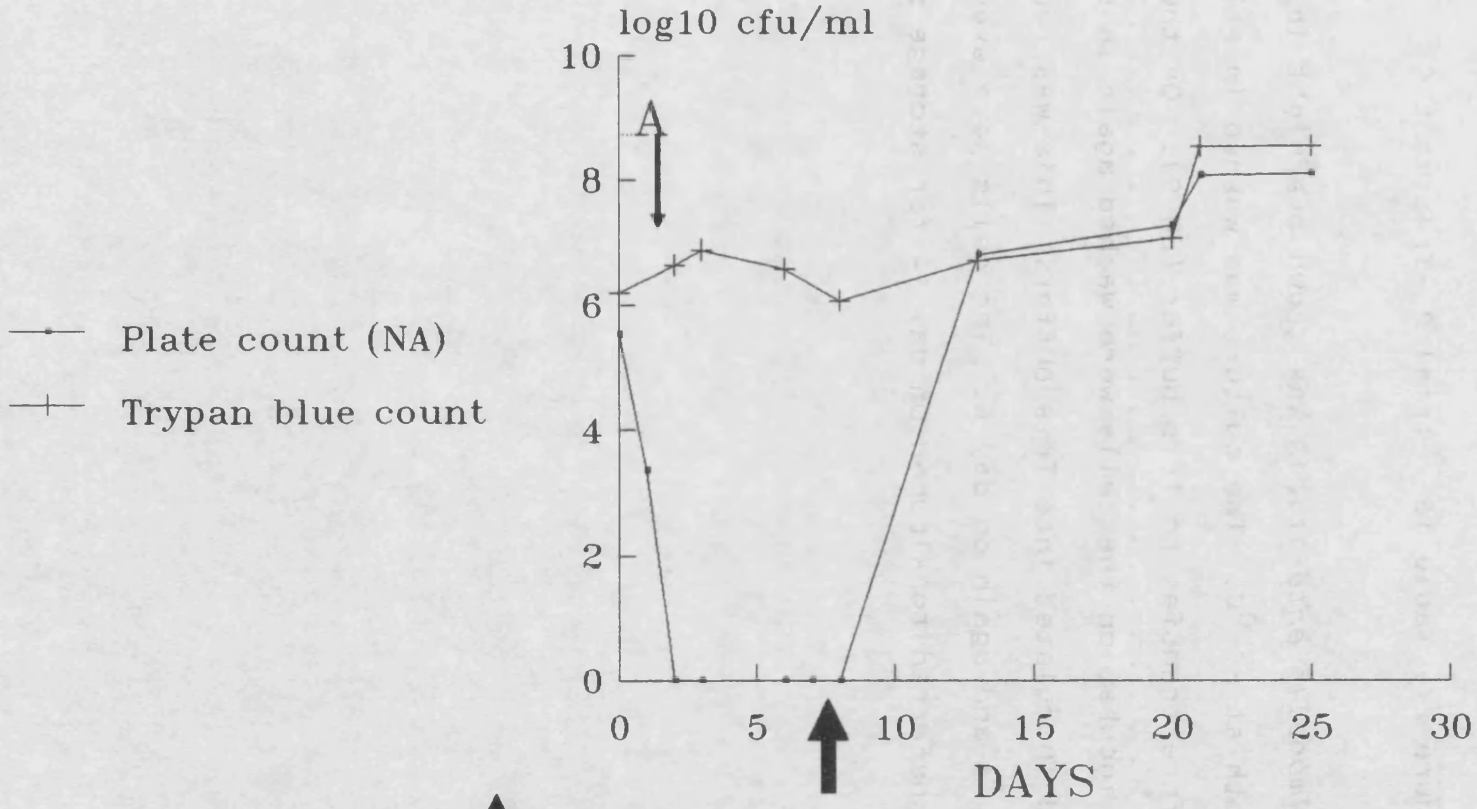


T = Transfer to Tris, A = Transfer to albumen. Results of 1 experiment

Figure 26. Table 15 - Trial 2 - Treatment A

Salmonella enteritidis was grown overnight in nutrient broth at 37 ^{0}C. The culture was washed in saline (x2) before transfer to Tris buffer (pH 9). On the second day of incubation the cells were washed again in saline (x2) and inoculated into albumen for storage at 37 ^{0}C.

Figure 26 The behaviour of *S.enteritidis* in albumen at 37°C following stress in Tris buffer (pH 9)

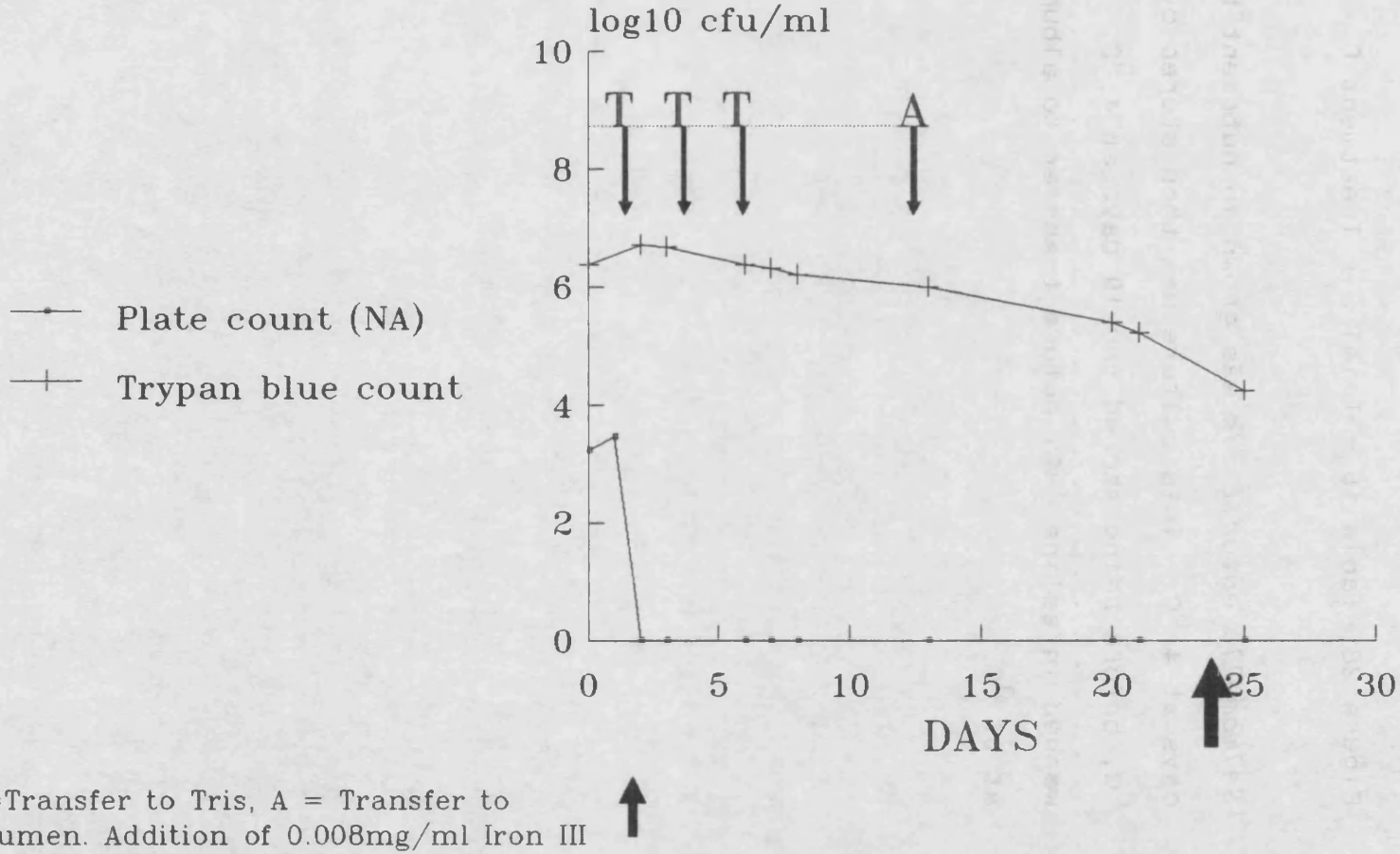


A =Transfer to albumen,
Addition of 0.008mg/ml Iron III
Results of 1 experiment

Figure 27. Table 15 - Trial 2 -Treatment C

Salmonella enteritidis was grown overnight in nutrient broth at 37 ^{0}C. The culture was washed in saline (x2) before transfer to Tris buffer (pH 9). On the second day of incubation the cells were washed again in saline (x2) and inoculated into Tris buffer. This was repeated on day 4 and again on day 6. The cells were eventually transferred to albumen on day 14 for storage at 37 ^{0}C.

Figure 27 The behaviour of *S.enteritidis* in albumen at 37°C following stress in Tris buffer (pH 9)

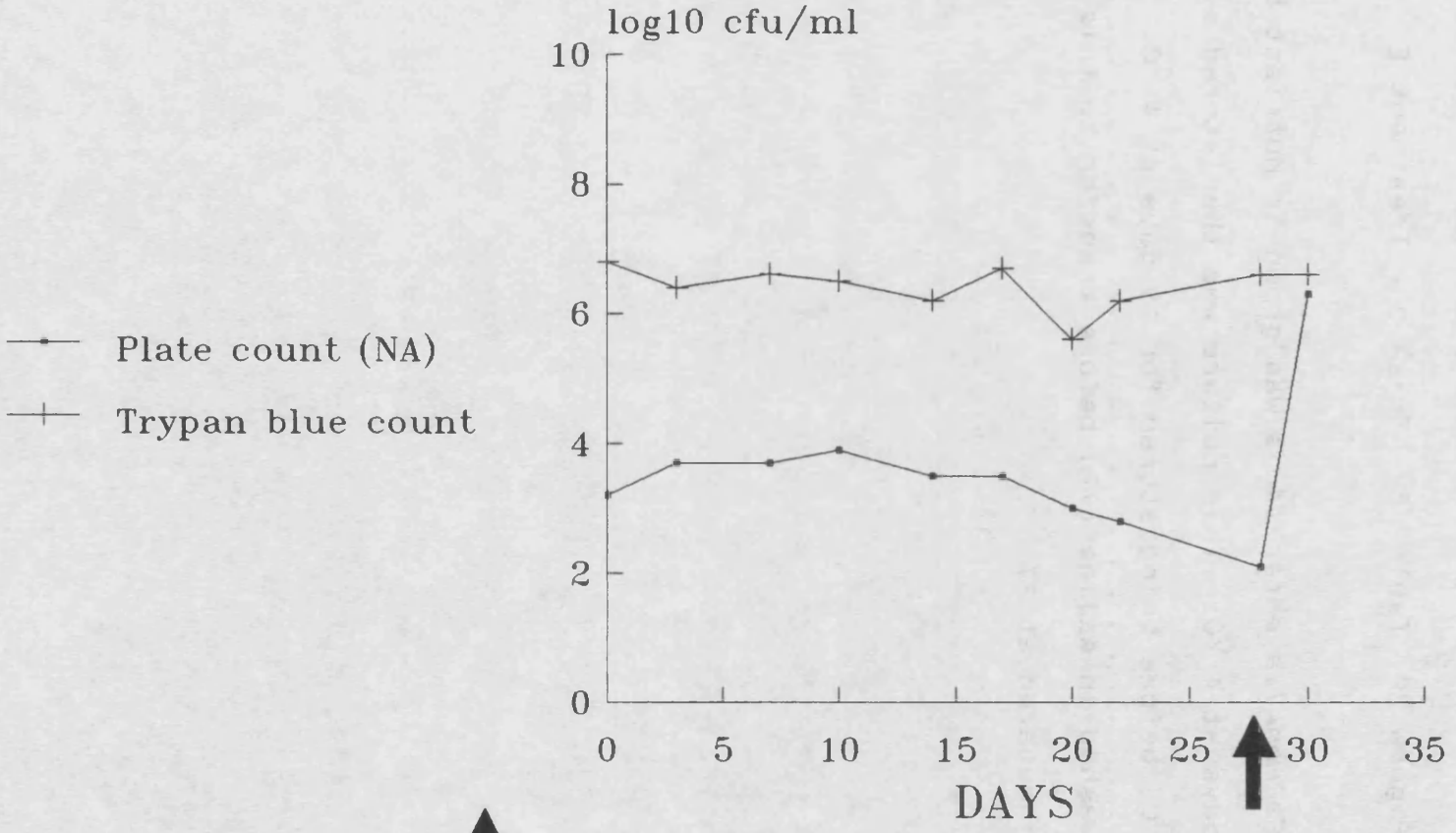


T =Transfer to Tris, A = Transfer to albumen. Addition of 0.008mg/ml Iron III
Results of 1 experiment

Figure 28. Table 15 - Trial 3 - Treatment D

Salmonella enteritidis was grown in nutrient broth for 9 days at 4 ^{0}C. This culture was then stored overnight at 37 ^{0}C, before being stored for 19 days at 4 ^{0}C. The cells were washed in saline (x2) before transfer to albumen incubated at 37 ^{0}C.

Figure 28 The behaviour of stressed *S.enteritidis* in albumen *in vitro* at 37 °C

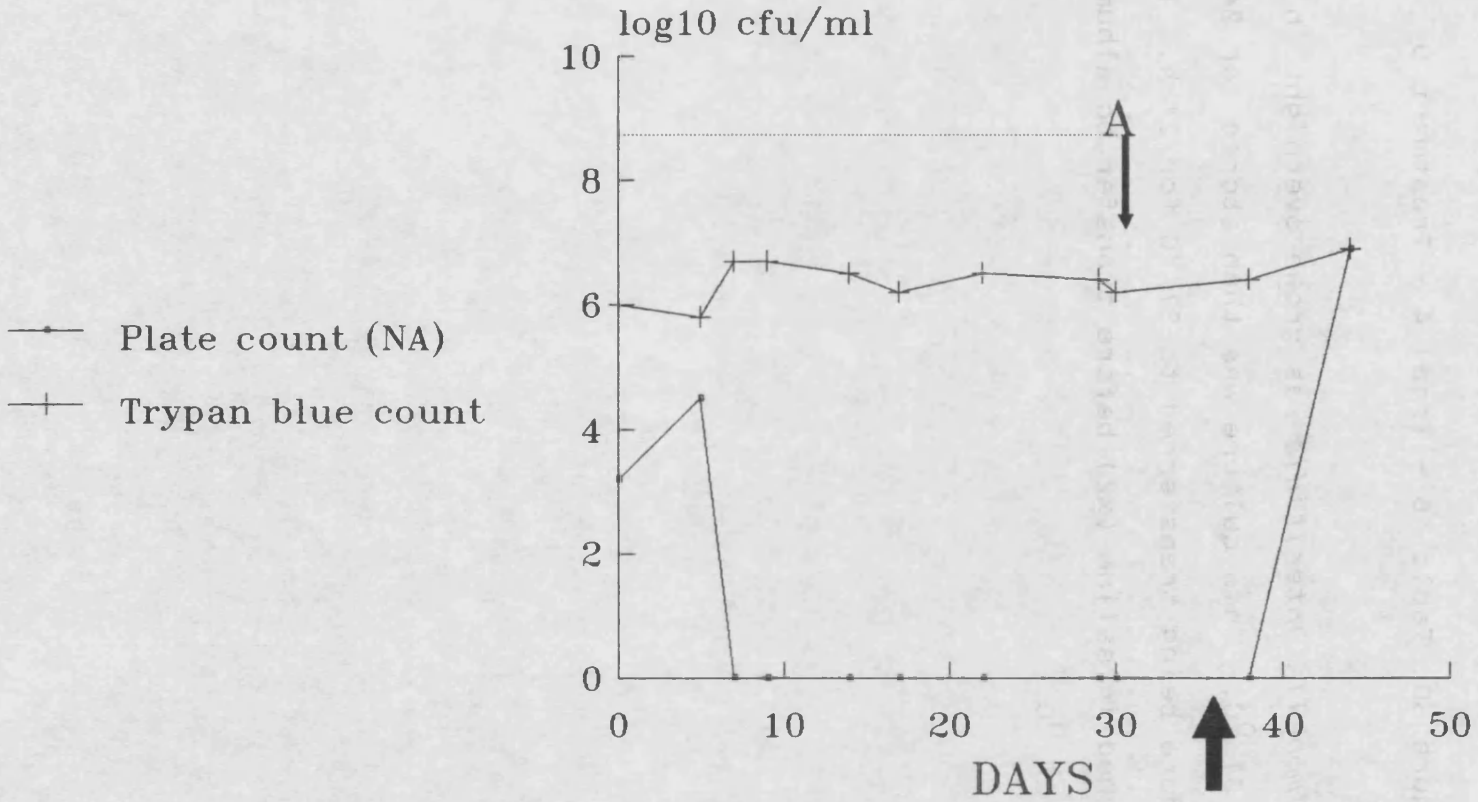


Results of 1 experiment

Figure 29. Table 15 - Trial 3 - Treatment E

Salmonella enteritidis was grown in nutrient broth for 9 days at 4 ^{0}C. This culture was then stored overnight at 37 ^{0}C, before being stored for 19 days at 4 ^{0}C. The cells were washed in saline (x2) before transfer to Tris buffer (pH 9) incubated at 37 ^{0}C.

Figure 29 The behaviour of *S.enteritidis* in albumen at 37°C following stress in Tris buffer (pH 9)

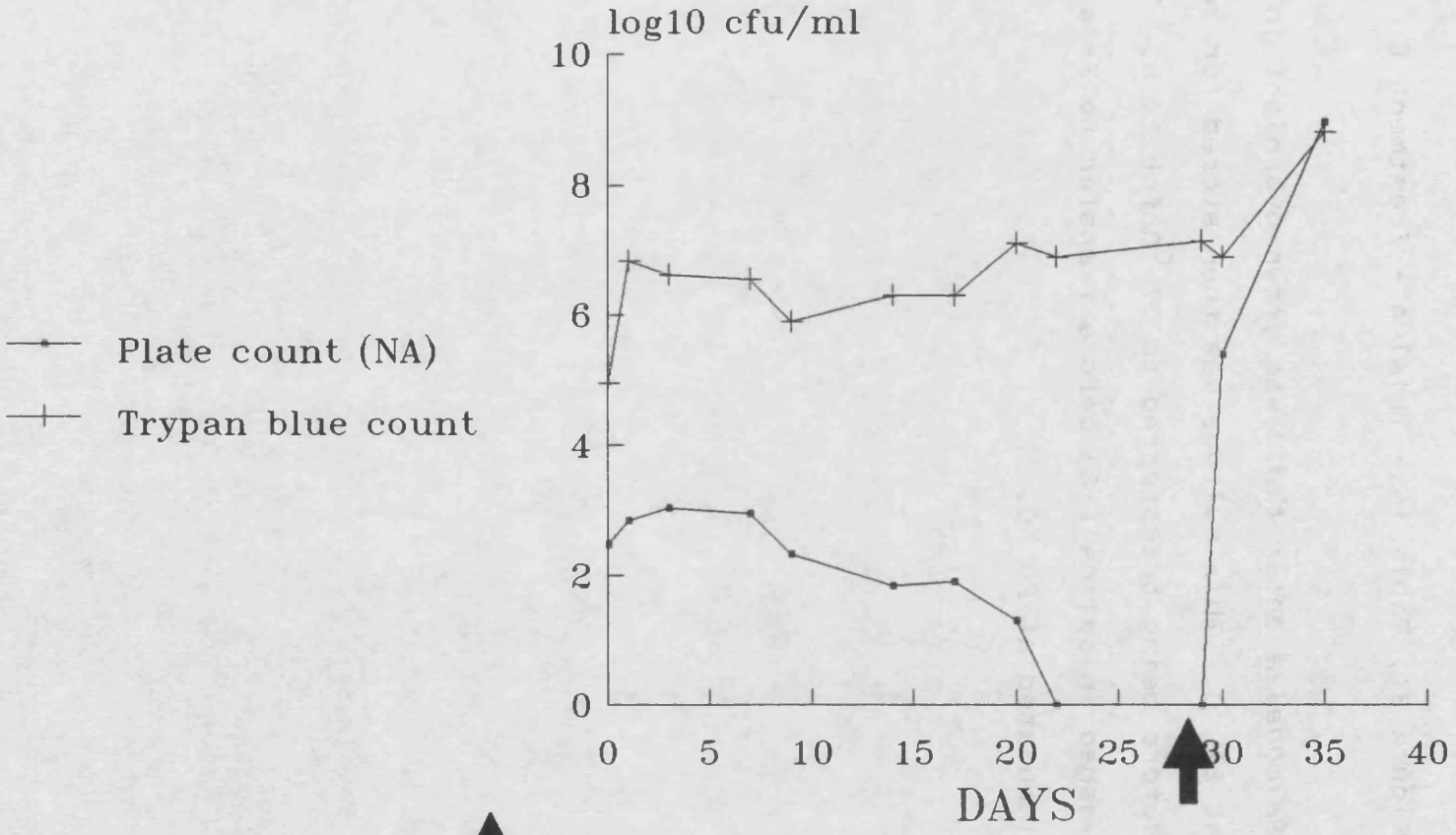


A = Transfer to albumen,
Addition of 0.008mg/ml Iron III
Results of 1 experiment

Figure 30. Table 15 - Trial 4 - Treatment D

Salmonella enteritidis was grown overnight in nutrient broth at 37 ^{0}C. This culture was then stored for 24 h at 44 ^{0}C, before being transferred to 37 ^{0}C for 24 h. The cells were washed in saline (x2) before transfer to albumen incubated at 20 ^{0}C.

Figure 30 The behaviour of stressed *S.enteritidis* in albumen *in vitro* at 20 °C

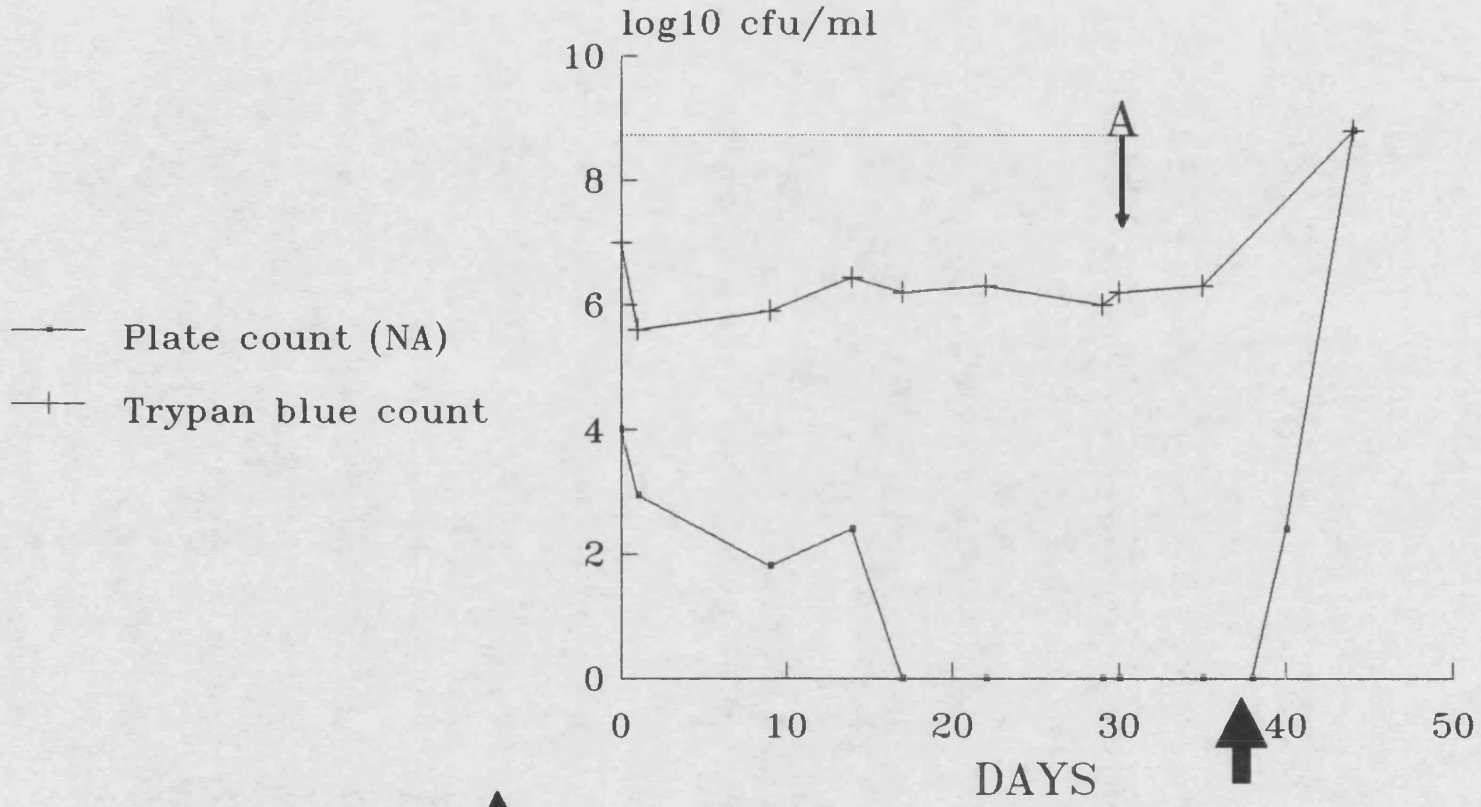


Results of 1 experiment

Figure 31. Table 15 - Trial 4 - Treatment E

Salmonella enteritidis was grown overnight in nutrient broth at 37 ^{0}C. This culture was then stored for 24 h at 44 ^{0}C, before being transferred to 37 ^{0}C for 24 h. The cells were washed in saline (x2) before transfer to Tris buffer (pH 9) incubated at 20 ^{0}C.

Figure 31 The behaviour of *S.enteritidis* in albumen at 20°C following stress in Tris buffer (pH 9)



A = Transfer to albumen
Addition of 0.008mg/ml Iron III
Results of 1 experiment

SECTION 3

A study to determine if chemotaxis plays a role in the generalized infection of the egg contents

The results presented in the previous sections indicated that salmonellas whether stressed or not remained quiescent or on some occasions multiplied sluggishly in albumen. With very large inocula (>10^6 cfu/ml), growth of unstressed cells occurred. Evidence was obtained also in support of the generally held view that iron deprivation by ovotransferrin was the major cause of bacteriostasis. These findings support the contention of Clay and Board (1991), namely that the initial salmonella contaminants of the albumen in whole eggs remain quiescent until some event induces their growth. They surmised that contact of contaminants and yolk was the event that triggered growth. In practice they could not distinguish between contact due to yolk movement, chance collision between contaminants in the albumen and the yolk or a combination of both events. The persistence of motility by non-growing salmonellas in albumen was highlighted in the present study (Table 11). Thus another factor, motility, needs to be taken into account. In practice motility could well cause one to question the use of the word "chance" in a discussion of the course of bacterial infection of eggs. Others have held different views on the course of events initiating gross contamination of the albumen. Brooks (1960) claimed that a change (uncharacterized) in the shell membranes triggered growth of contaminants in these structures and that growth

"spilled over" into the albumen. Recently Humphrey *et al.* (1991) surmised that nutrients diffusing from the yolk may trigger growth of salmonellas in the albumen. As was evident in the Literature Review, there is very limited information about the diffusion of small molecules outward from the yolk. Nevertheless even a limited amount of diffusion would create a gradient that might well elicit a chemotactic response. This section is concerned with Brooks' (1960) contention of a change in the shell membrane promoting bacterial growth, as well as bacterial motility and possible chemotaxis.

MATERIALS AND METHODS

A study of *S.enteritidis* in the shell membrane suspended in albumen alone or in whole egg *in vitro*

The air cells of eggs were located by candling. The shell was swabbed with 70% (v/v) ethanol, a small hole drilled (Minicraft Drill MB0502) in the shell at this site and 0.1 ml of a ca. 10^3 cell suspension injected on to the air cell membrane. The hole was sealed with paraffin wax and the eggs left at room temperature for ca. 3 h. All the liquid was absorbed into an egg's contents within 20 min of being applied to the shell membrane. An eggshell was then wiped with ethanol, the shell cracked and the contents poured gently into a sterile specimen pot (Sterilin, 250 ml). The inoculated inner membrane of the air cell was excised and placed in the albumen alone or in the albumen of whole egg in a sterile container. In the latter case, care

was taken to ensure that the inoculated membrane was as far away as possible from the yolk.

The inoculated samples were stored at 4, 20 or 30 ^{0}C. Duplicate samples were tested at frequent intervals for the presence of *S.enteritidis*. Viable counts of albumen and yolk were obtained on XLD. Care was taken when extracting samples of yolk to include as little albumen as possible. The membrane was plated directly onto XLD or placed in Ringers solution (¼ strength, Lab M) containing glass beads, mixed thoroughly and sonicated (Cleaning Sonicator, Dawes Instruments Ltd, USA) for 4 min before plating of dilutions on XLD. This process did not reduce bacterial viability.

Initially a range of inocula (ca. 10^2, 10^3, 10^4 and 10^5 salmonellas/membrane) suspended in albumen *in vitro* and stored at 20 ^{0}C was tested.

A study to determine whether or not the yolk induces a chemotactic response in *S.enteritidis* and *Pseudomonas putida*

An overnight culture of *S.enteritidis* in nutrient broth (37 ^{0}C) containing 0.05% (w/v) Calcofluor (Fluorescent Brightner Number 28, Aldrich), as used by Paton and Ayres (1964), or *Pseudomonas putida* was washed in saline and spun down (2000 g x 10 min). Sterile water agar (7-9 g/l agar; Lab M) was seeded with ca. 10^9 cfu/ml and allowed to gel in a Petri dish. Plugs (7 mm diameter) were removed from the agar using a sterile cork borer.

The shell of an egg (<2 days old or stored at 30 ^{0}C for >7 days) was sterilized with 70% (v/w) ethanol, the shell cracked and the contents poured gently into a 100 x 100 mm

square plate (Sterilin). An agar plug, either control or inoculated, was placed in each corner of the dish such that inoculated plugs were diametrically opposed. To prevent water loss Petri dishes were stored in plastic bags at 4 or 20 ^{0}C. Movement of organisms out from the plugs was followed by examination of an egg under UV light (Camag - Camlab, 350 nm), microscopically (a loopful examined with x1000 phase contrast) and by the plate count method with XLD.

Chemotaxis was investigated further by the protocol outlined in Figure 32. *Salmonella enteritidis* grown overnight in nutrient broth containing Calcofluor (0.05% w/v) was inoculated using a syringe (0.5 ml - ca. 10^{9} cfu/ml) into the glass tube (internal diameter 3mm, length 33 cm - Figure 32), such that the organisms were located at the bottom of the measuring cylinder but not in the agar at the orifice of the tube. The apparatus was stored at 20 ^{0}C, the progress of the organisms followed under UV light (350 nm) and recorded photographically. Organisms were isolated from the agar and checked serologically.

RESULTS

In initial experiments a range of inoculum sizes in the excised membrane of the air cell of eggs was studied with albumen alone and incubation at 20 ^{0}C (Figure 33). It is evident from this Figure that the survival of the organisms in the air cell membrane was influenced by inoculum size. Thus inocula of ca. 10^{4}, 10^{3} and especially 10^{2} cfu/membrane showed a progressive decrease in the number of

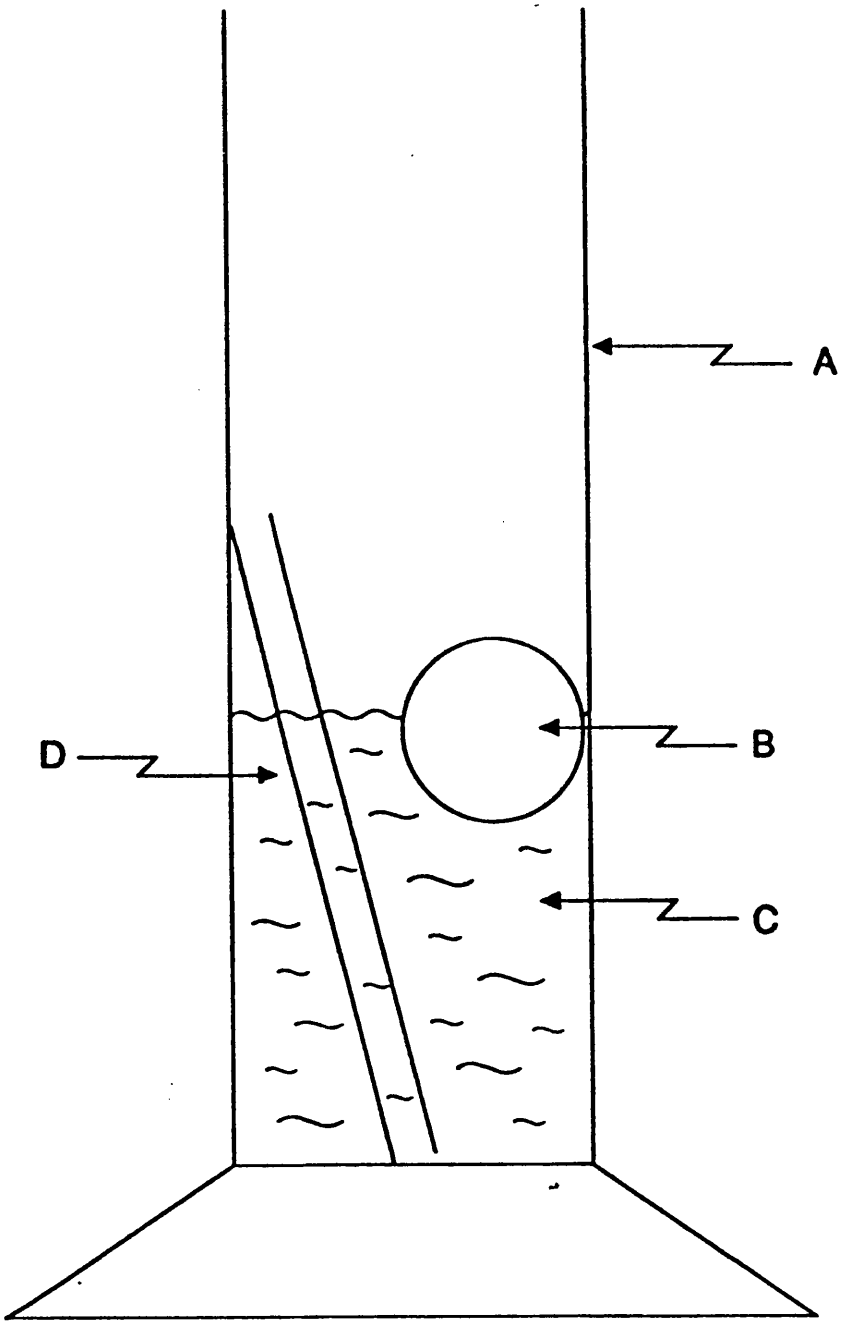


Figure 32. A diagramatic representation of the apparatus used to follow the course of *S.enteritidis* to the yolk

(a) One litre plastic measuring cylinder, filled to 400 ml.

(b) Whole yolk or a ping-pong ball (control - drilled, filled with water and adjusted to the weight of the yolk).

(c) Water agar 0.2% (w/v) pure agar (Lab M).

(d) Sterile glass tube - bottom edge chamfered.

viable organisms over 10 days incubation. Very slight growth of the organism in membranes occurred, however, with an inoculum of *ca*. 10^5 cfu/membrane. There was a modest increase in the number of viable organisms in the albumen when an inoculum of this size was used (Figure 33b).

Two temperatures, 4 ^{0}C and 25 ^{0}C, were used in two preliminary experiments - the latter temperature was selected as a compromise between 20 and 30 ^{0}C as used in previous experiments. *Salmonella enteritidis* was present in very low numbers in the excised inner membrane of the air cell membrane suspended in albumen for upwards of 17 days at 4 or 25 ^{0}C (Table 16). Microscopic observations of the albumen taken on every sampling occasion failed to detect organisms and no viable organisms were recovered on XLD from the albumen at 4 or 25 ^{0}C.

In these experiments the membranes contained initially *ca*. 10^3 salmonellas. The sonication of a membrane permitted counts of *ca*. 100 organisms/membrane to be determined. When the population fell below this level, the membrane was smeared across and left on the surface of XLD agar. Towards the end of the experiment, growth occurred mainly beneath the membranes taken from albumen stored at 4 ^{0}C.

In a further experiment *ca*. 10^3 cfu/membrane was again used as the inoculum, incubation temperatures of 4, 20 or 30 ^{0}C were used with 4 eggs being tested on each sampling occasion. Viable organisms were present, but in low numbers only, in the albumen *viz à viz* those on the membrane until day 4 at 4 ^{0}C (Figures 34a & b). This was the case also (Figures 34c & d) in a repeat experiment. On XLD agar

viable organisms grew beneath the membranes recovered from albumen stored at 4 ^{0}C for upto 37 days.

At 20 ^{0}C (Figures 34e-h), organisms were detected in the albumen on the majority of sampling occasions. The number of organisms recovered from the membrane decreased progressively during the 37 days of observation, but organisms were always present beneath membranes resting on XLD agar. This trend was evident also at 30 ^{0}C (Figures 34i-l). When compared with results obtained at 20 ^{0}C, organisms were recovered on fewer occasions, but in comparable numbers from the albumen incubated at 30 ^{0}C. These results demonstrate that the shell membrane did not change with time such that the growth of salmonellas was favoured, as postulated by Brooks (1960).

Salmonella enteritidis was inoculated onto the air cell membrane of whole eggs. The membrane was excised from broken out eggs and placed in the albumen of the corresponding egg's contents contained in a sterile container (250 ml, Sterilin). Nevertheless with eggs stored at 4 ^{0}C, the organisms remained viable (as shown by growth beneath pieces of membrane on XLD agar) in the membrane for 37 days. With time the number of organisms present in the membrane at 4 ^{0}C diminished to levels below that (100 cfu/ml) detectable by dilution and plating methods. This situation obtained from day 19 onwards. The organisms were detected in the albumen by plate counts on day 23 in both sets of experiments (Figures 35a-d), but in numbers too few to be observed microscopically (limit of detection $>10^{5}$ cfu/ml). *Salmonella enteritidis* was not isolated from the

yolk on any sampling occasion with storage at 4 ^{0}C (Figures 35e & f).

At 20 ^{0}C, there was a gradual increase in the number of organisms found in the membrane and in the albumen such that by day 26 (Figures 35g-j) the populations were >10^{7} cfu/ml albumen. The yolk was infected with small numbers of organisms on day 3 (Figures 35k-l) but large populations were not detected until day 26. When the membrane drifted in the albumen and became lodged on the yolk, then gross contamination of the albumen and yolk occurred much earlier - usually within 12 days - than noted above .

The rate and extent of systemic infection of the albumen and yolk at 30 ^{0}C was similar to that at 20 ^{0}C, with the number of organisms exceeding *ca*. 10^{7} cfu/ml in the membrane, albumen and yolk by day 26 (Figures 35m-r). Yolk infection was detected in one trial on the day following inoculation and in another on day 3. The number of organisms present increased gradually over the 37 days of incubation at which time the numbers exceeded 10^{7} cfu/ml. Microscopic observations failed to detect the presence of organisms in the albumen until the number of cells had reached >10^{5} cfu/ml.

The results presented above refute the suggestion of Brooks (1960) that some change in the membrane promotes bacterial growth and systemic infection of egg contents. They do, however, support the contention of Clay and Board (1991) that the yolk plays a role in triggering bacterial growth. Indeed the results presented in Figure 35 are markedly similar to those obtained with whole eggs by these

workers. The exact contribution of the yolk, however, has yet to be determined. For example, is the yolk merely a depot of nutrients or does the outward diffusion of nutrients establish a gradient and thereby invoke a chemotactic response in the initial contaminants of the albumen ? The following section presents results of experiments which sought to discover if organisms in the albumen express a chemotactic response.

Salmonella enteritidis grown in nutrient broth containing Calcofluor was used to seed plugs of water agar (>10^6 cfu/plug). A large inoculum was used in order to ensure that the passage of the organisms was easily observed. These were placed in the albumen at the greatest possible distance from the yolk of whole eggs. Broken out eggs were held in square Petri dishes, at 4 or 20 ^{0}C. Initially an attempt was made to follow the passage of the organism photographically using UV light. Fluorescence occurred initially in the plugs. It was never detected in the albumen. In practice photographs showed only clouding due to large numbers (ca. 10^6 cfu/ml) of organisms in the albumen. No organisms were observed microscopically in the albumen until the numbers exceeded ca. 10^5 cfu/ml. This event coincided with the onset of a slight clouding in the albumen.

At 4 ^{0}C *S.enteritidis* occurred in low numbers in all of the albumen samples for upto 18 days of incubation (Figures 36a & c). The organism was never isolated from the yolk on any sampling occasion (Figures 36b & d). At the higher temperature (20 ^{0}C), the number of *S.enteritidis* in

the albumen increased progressively throughout incubation. The organisms were detected in the yolk on day 3, and by day 18 the level of contamination exceed ca. 10^6 cfu/ml (Figures 36e-h).

A comparable experiment was conducted with aged eggs (stored at 30 ^{0}C for 7 days before inoculation). There was no definition of albumen structure in these eggs. In those stored at 4 ^{0}C, organisms were detected in low numbers in albumen surrounding the yolk by day 6. None was ever detected in the yolk. At 20 ^{0}C organisms were present in the albumen and the yolk on the day following inoculation. Large populations obtained by day 6. The extent of the growth of the organism was such that it was easily monitored microscopically.

Evidence of possible chemotactic response by *S.enteritidis* was sought with the apparatus illustrated in Figure 32. The organisms were placed at the bottom of the glass tube having a chamfered end. From this location the organisms were free to move into the water agar in the measuring cylinder. From Plates 1 & 3 it is evident that with time the organisms formed a dense cloud around the yolk. The presence of *S.enteritidis* at this site was confirmed serologically. A cloud did not develop in the control containing a ping-pong ball (Plate 2 & 3). The experiment was repeated on 4 occasions and the same result was obtained on every occasion. Serology was used to confirm the presence of the organism on each occasion.

Failure to follow the passage of *S.enteritidis* grown in a medium containing Calcofluor led to the use of *Pseudomonas*

putida (isolated from a rotten egg) in further work. The behaviour of this naturally fluorescent bacterium in eggs is the same as that of *S.enteritidis* (Dolman and Board 1992). Plate counts revealed that there was a progressive increase in the number of pseudomonads in the albumen at 4 ^{0}C (Figures 37a & c). The yolk was contaminated by day 13 (Figures 37b & d). At 20 ^{0}C, *Ps.putida* was present in the yolk on day 11 in one egg (Figure 37h) and day 13 in the remainder (Figures 37f & h). It was notable that *Ps.putida* never attained populations as large as those of *S.enteritidis* in an equivalent time span (Figure 36). Progressively throughout the course of this experiment, the albumen became increasingly contaminated with large numbers of organisms (Figures 37e & g). Microscopic observations monitored this increase of contamination in the albumen once the number of organisms attained ca. 10^{5} cfu/ml.

The progress of infection of eggs inoculated with *Ps.putida* and stored at 4 and 20 ^{0}C is illustrated in Plates 4-14. It is evident that there was no detectable change in the appearance of the egg albumen until day 16 at 4 ^{0}C and day 13 at 20 ^{0}C at these times fluorescence was a feature. Indeed in all of the eggs tested the demonstration of fluorescence and the numbers of organisms in the albumen (exceeding 10^{5} cfu/ml) were coincident (Figures 37a, c, e and g). The pattern of events recorded in Plates 4-14 leads me to propose the following sequence of events that culminates in heavy infection of the contents and, with an appropriate organism, addling of eggs (Figure 38).

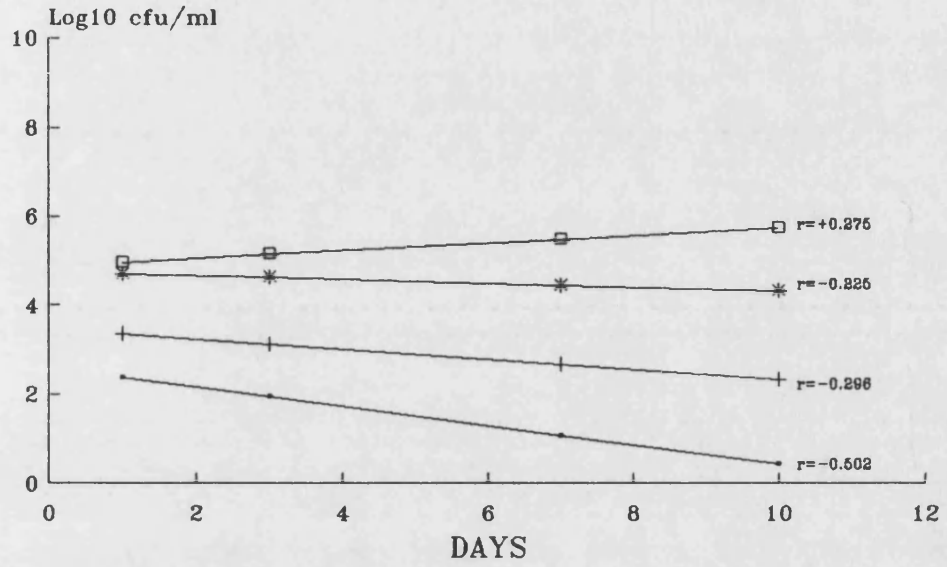
(1) Organisms present in the albumen (or the shell membranes) persist in a nutrient deprived state until there is either a chance or more probably a chemotactically induced collision with the yolk. Indeed the experiment with the apparatus shown in Figure 32 and the square plates do demonstrate the possible contribution of chemotaxis to generalized infection of eggs. When collisions occur an abundant source of nutrients is made available to the organism. (2) The organisms are confined initially to the thin white surrounding the yolk. (3) With time organisms spread outwards into the albuminous sac. The contaminants appear to be contained until released by the deterioration of the albuminous sac as the egg ages. (4) This leads to gross infection of the entire egg contents. The rate of progress of infection is determined by the storage temperature. Indeed the effect of temperature on the rate of decay of the albuminous sac may well be more important than that of the temperature on the growth rate of the contaminant.

Summary

In summary from the results presented in this thesis one may conclude the following. If organisms such as salmonellas or pseudomonads gain access to the albumen, then they are able to (a) persist in a quiescent/somnicell state until induced to multiply by contact with the yolk or by nutrients diffusing from the yolk (Humphrey 1991), or (b) remain motile for a sufficient length of time to express a chemotactic response and make contact with the yolk.

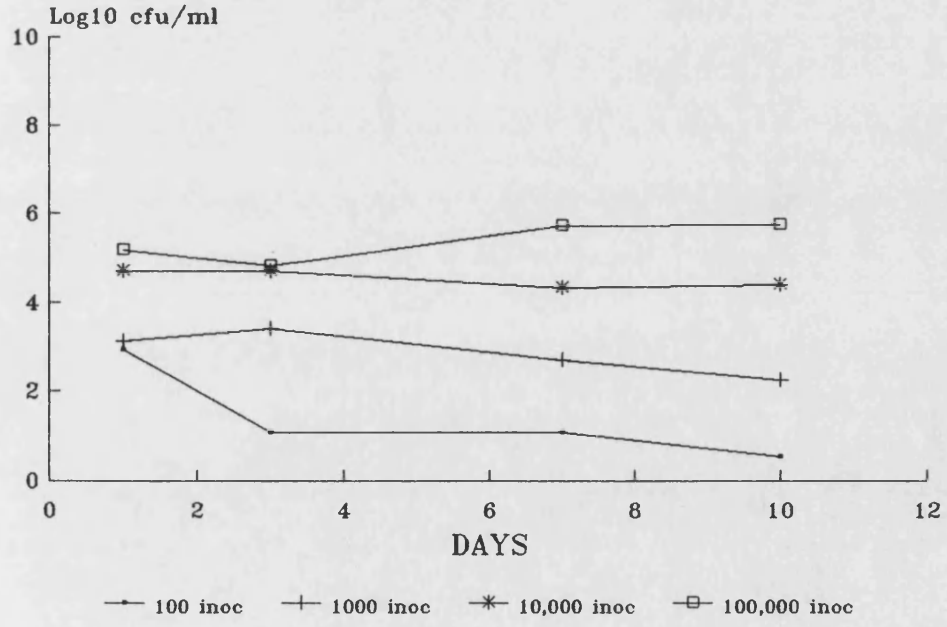
It is probable that gross contamination of an egg's contents is the result of a combination of 2 factors. Firstly the thinning of the albumen hence increasing mobility of the yolk favouring a collision between it and organisms, and secondly a chemotactic response that favours the movement of organisms towards the yolk.

Figure 33 Fate of different size inocula of *S.enteritidis* in the air cell membrane suspended in albumen *in vitro* at 20 °C



Regression of 5 eggs for each point

(a) - Albumen cfu/ml



Average of 5 albumen samples

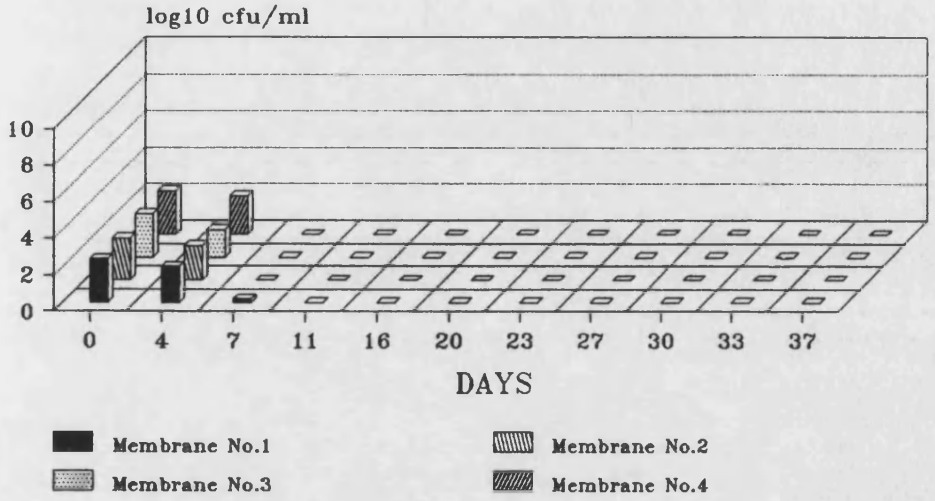
Table 16. The persistence of *S.enteritidis* PT 4 in a piece of shell membrane in albumen *in vitro*

Days	Persistence at 4 ^{0}C		Persistence at 25 ^{0}C	
	Membrane	Albumen	Membrane	Albumen
1	3.25*	-	3.38	-
3	2.32	-	3.31	-
6	1.70	-	3.18	-
8	1.0	-	3.10	-
10	+	-	3.0	-
13	+	-	2.74	-
15	+	-	2.48	-
17	+	-	2.35	-

* = log10 cfu/ml. Each is an average of 3 eggs
+ = organism grew beneath the membrane placed on XLD agar
- = no viable organisms grew from 0.1 ml of albumen plated on XLD or Nutrient agar

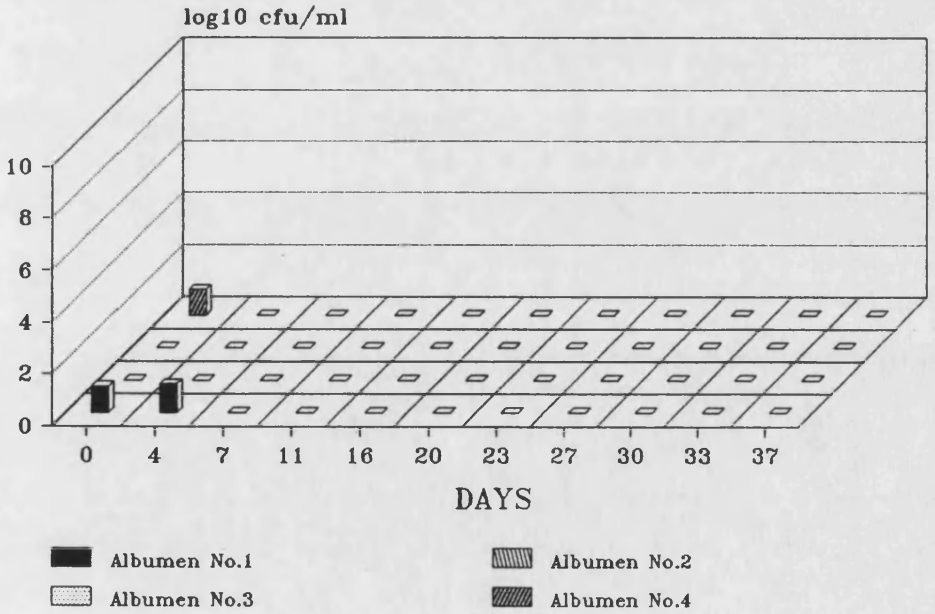
This experiment was done on 2 occasions with an initial inoculum of ca. 10^3 cfu/membrane.

Figure 34 The behaviour of *S.enteritidis* in the air cell membrane suspended in albumen (a) at 4°C. Membrane (Experiment 1)

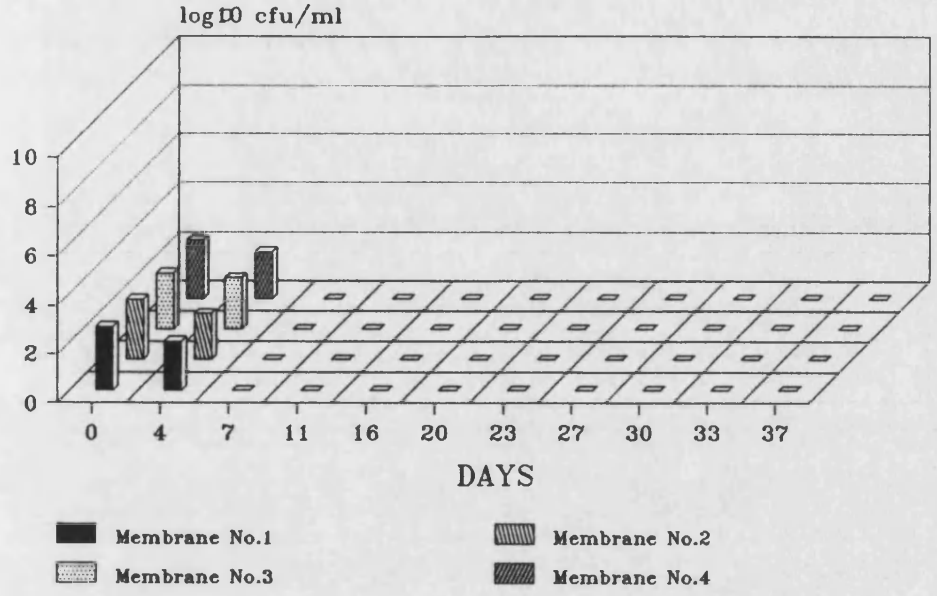


Growth occurred under the air cell membrane on every sampling occasion

(b) at 4°C. Albumen (Experiment 1)

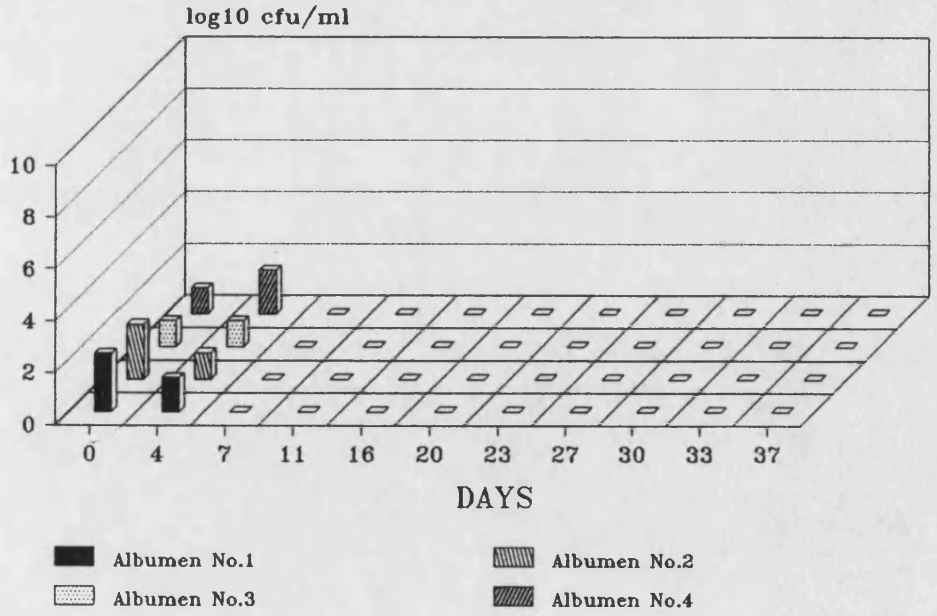


(c) at 4 C. Membrane (Experiment 2)

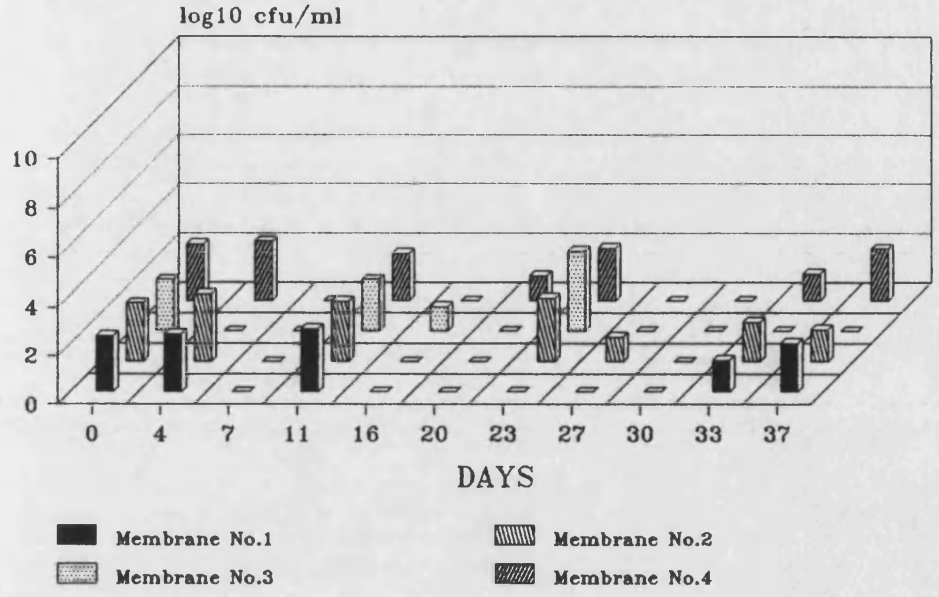


Growth occurred under the air cell membrane on every sampling occasion

(d) at 4° C. Albumen (Experiment 2)

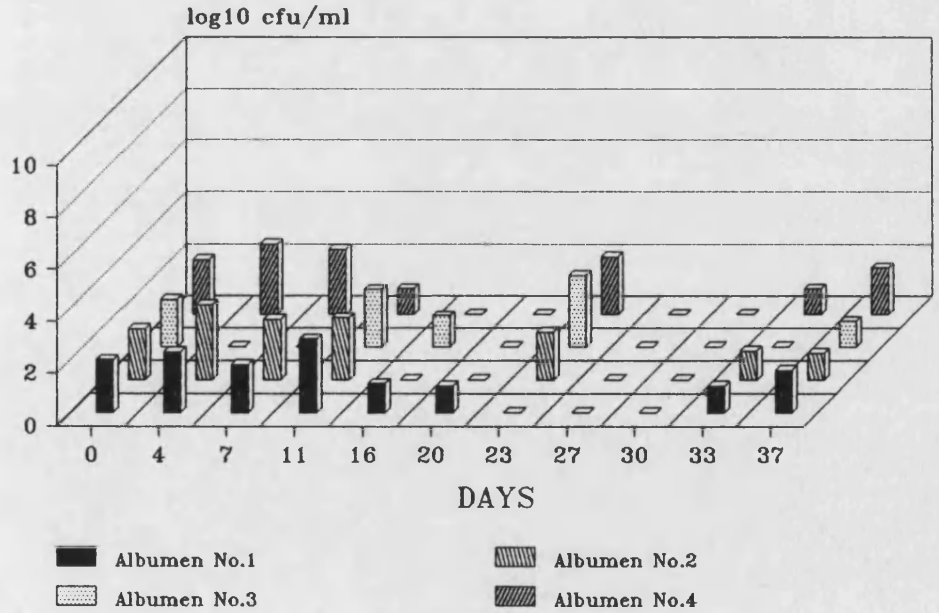


(e) at 20°C. Membrane (Experiment 1)

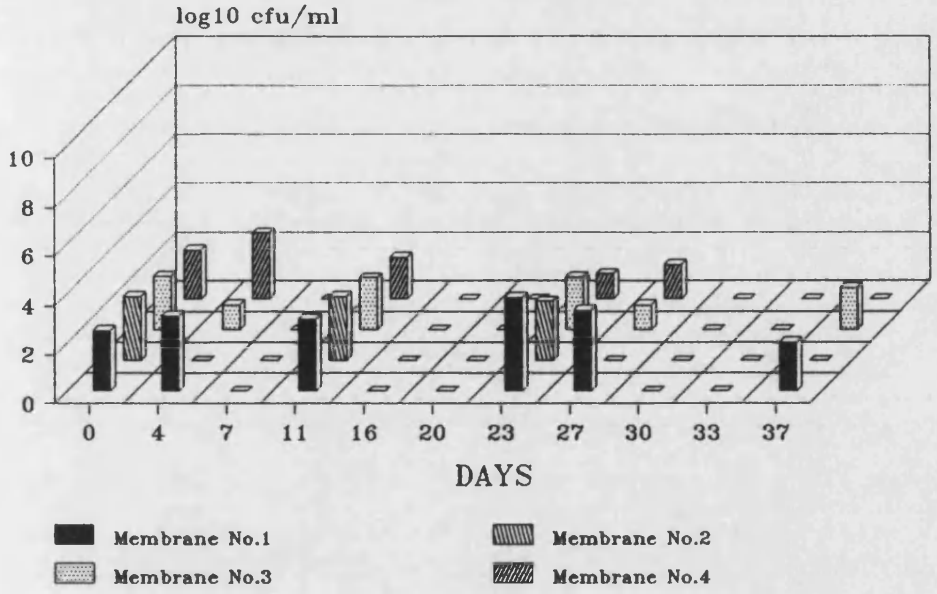


Growth occurred under the air cell membrane on every sampling occasion

(f) at 20°C. Albumen (Experiment 1)

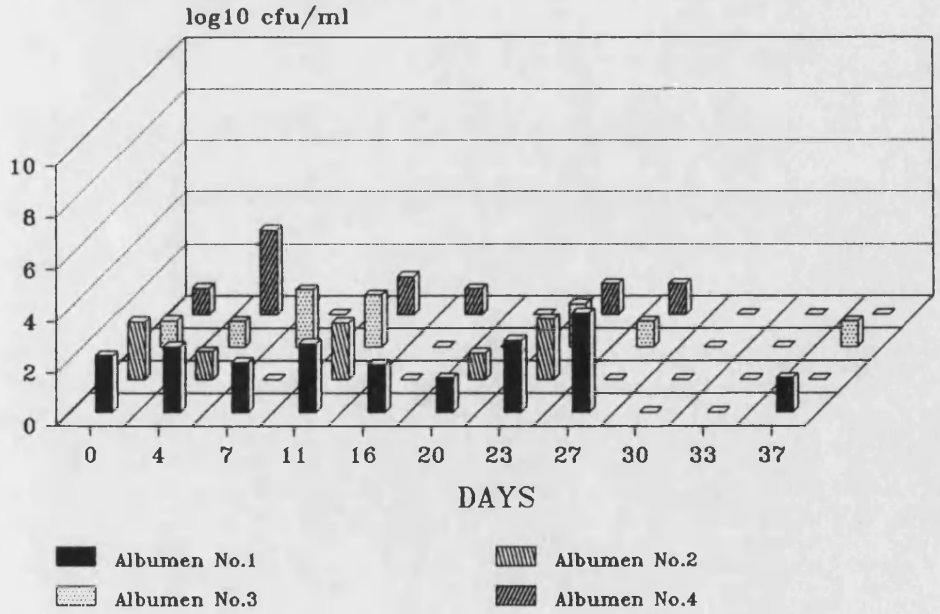


(g) at 20°C. Membrane (Experiment 2)

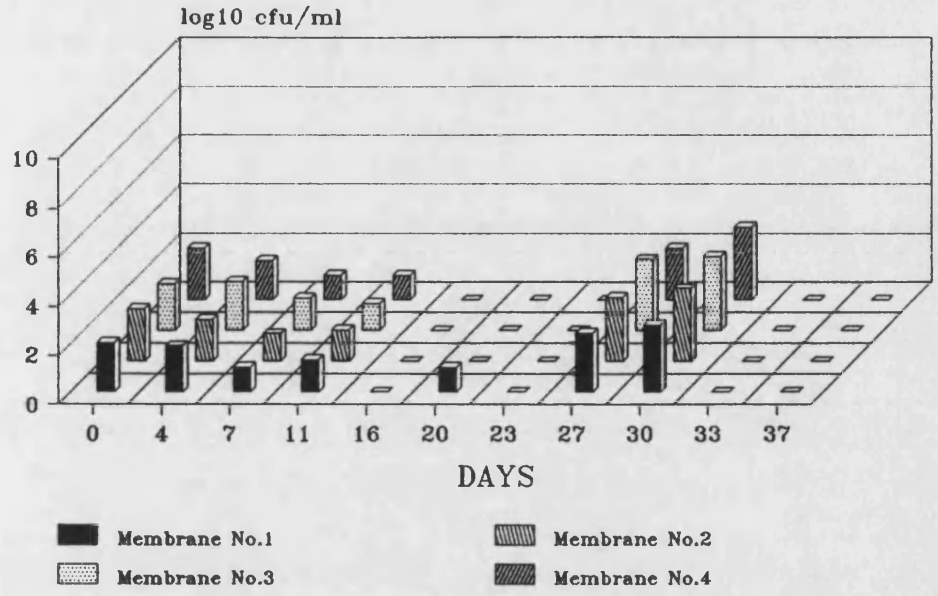


Growth occurred under the air cell membrane on every sampling occasion

(h) at 20° C. Albumen (Experiment 2)

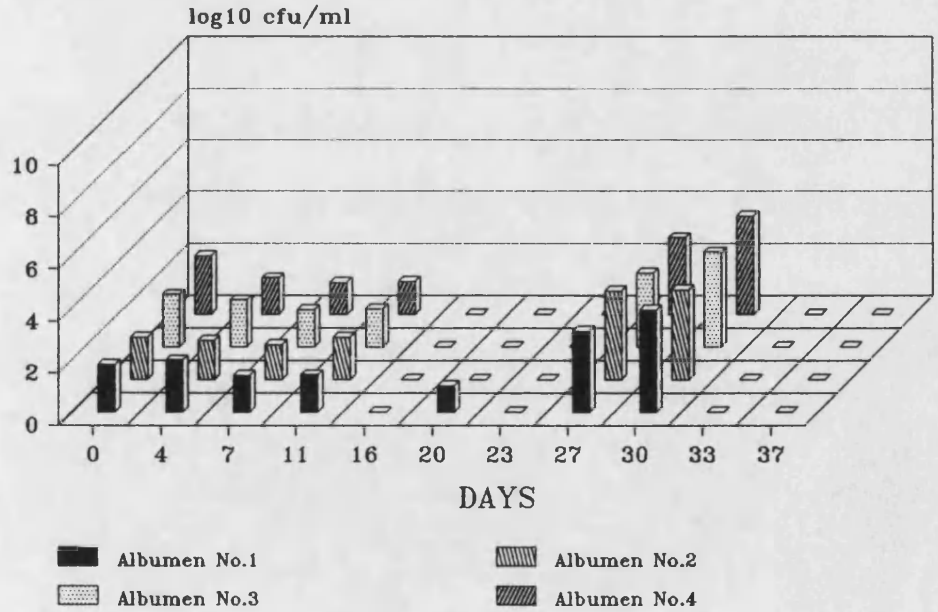


(i) at 30 °C. Membrane (Experiment 1)

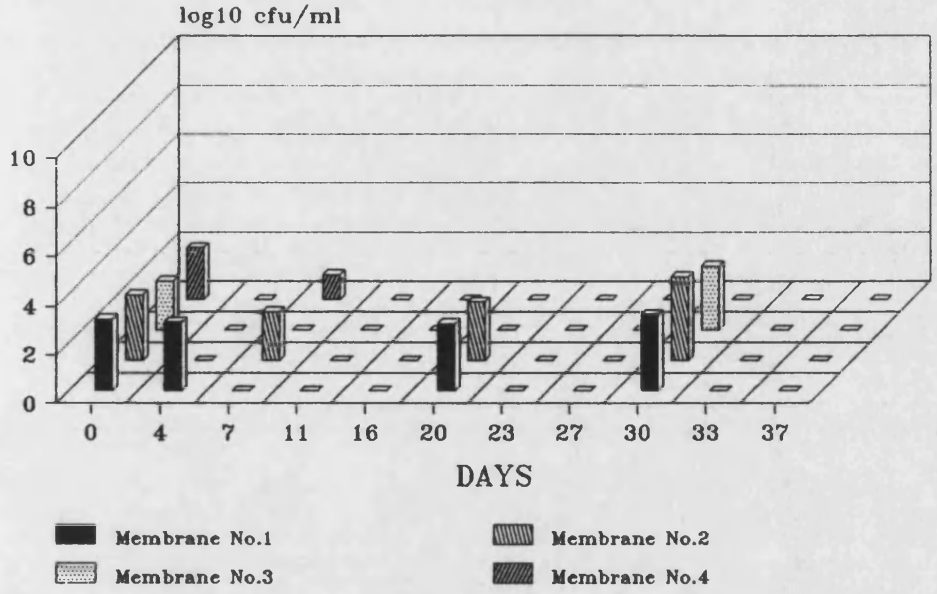


Growth occurred under the air cell membrane on every sampling occasion

(j) at 30 °C. Albumen (Experiment 1)



(k) at 30°C. Membrane (Experiment 2)



Growth occurred under the air cell membrane on every sampling occasion

(l) at 30°C. Albumen (Experiment 2)

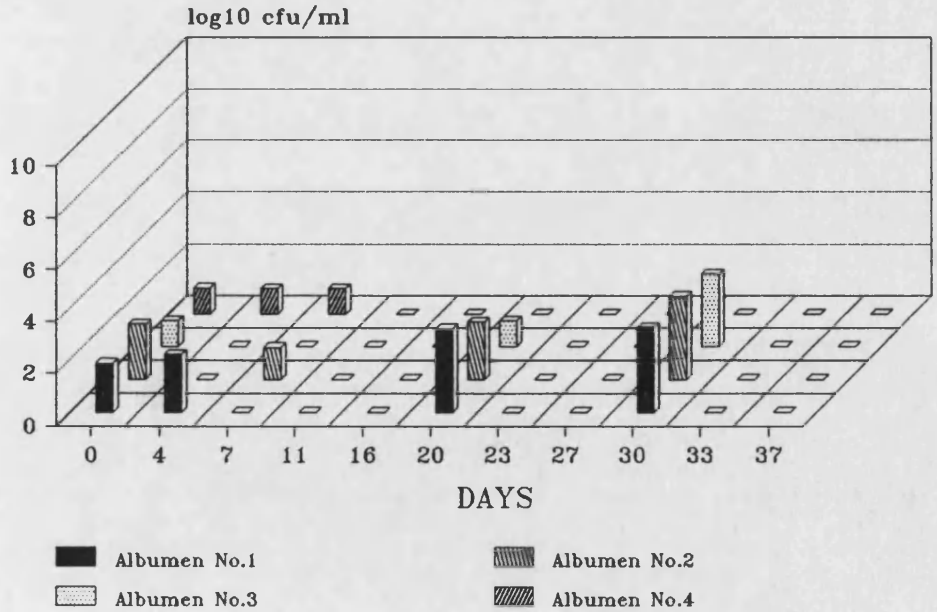
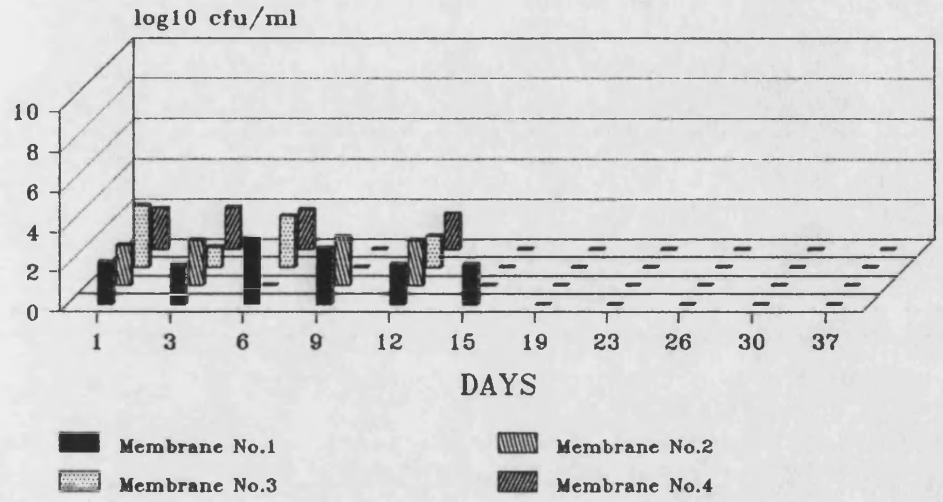


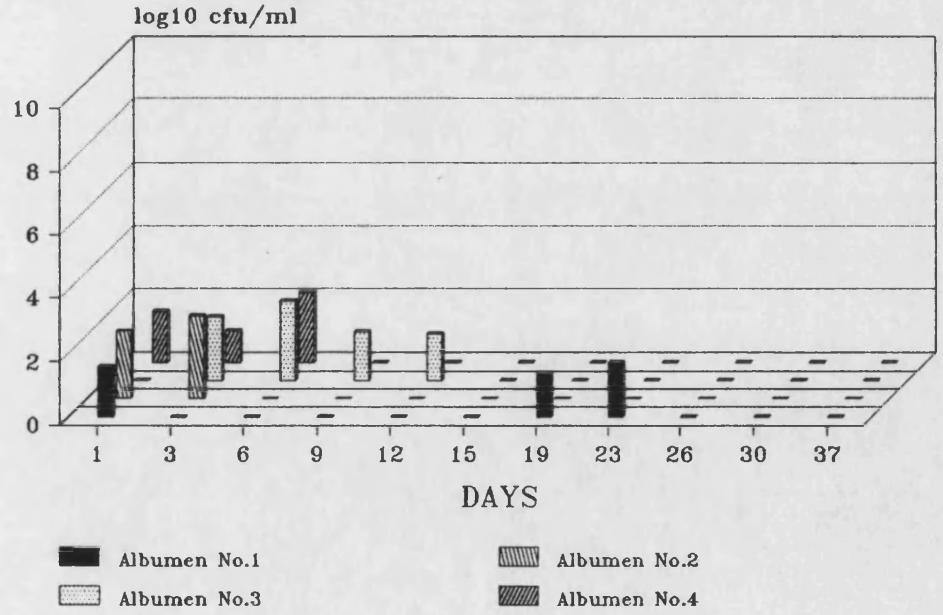
Figure 35 The fate of *S.enteritidis* in the air cell membrane suspended in whole egg

(a) at 4°C. Membrane (Experiment 1)

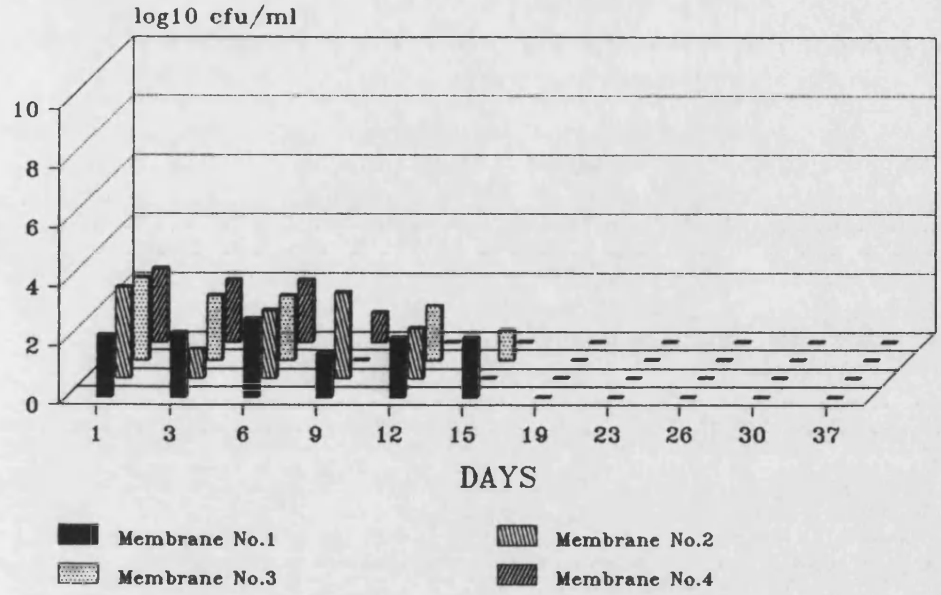


Growth occurred under the air cell membrane on every sampling occasion

(b) at 4°C. Albumen (Experiment 1)

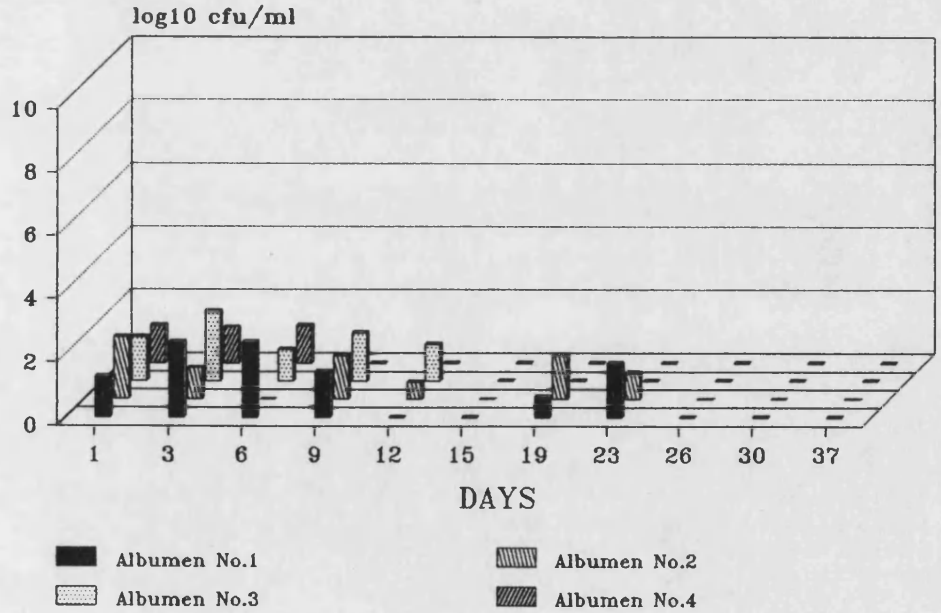


(c) at 4°C. Membrane (Experiment 2)

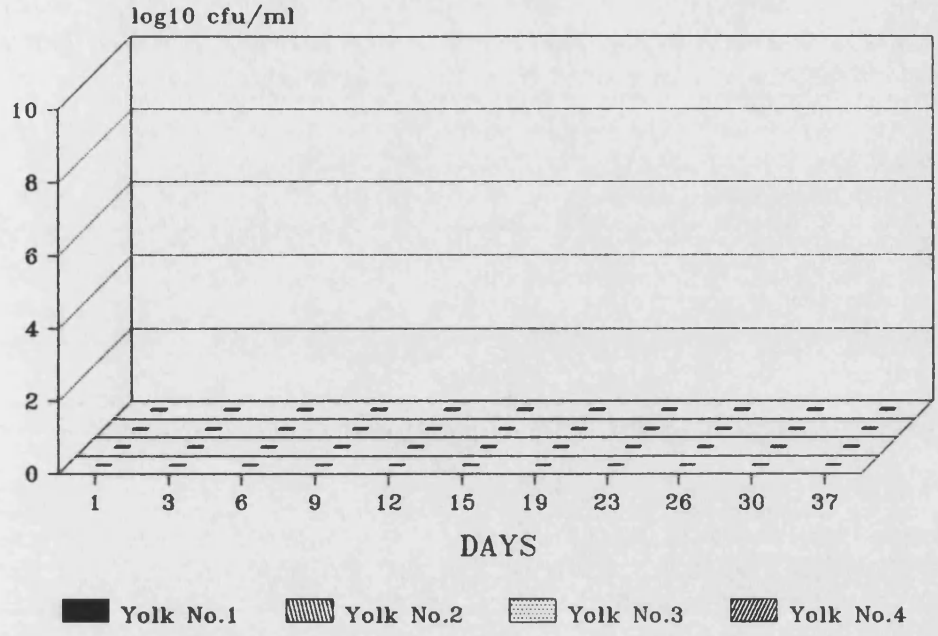


Growth occurred under the air cell membrane on every sampling occasion

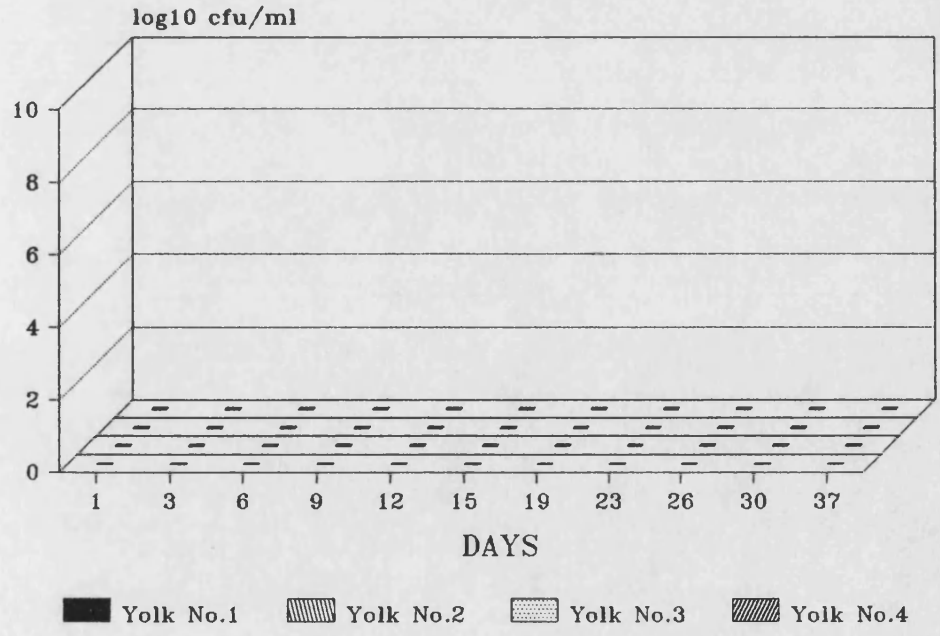
(d) at 4°C. Albumen (Experiment 2)



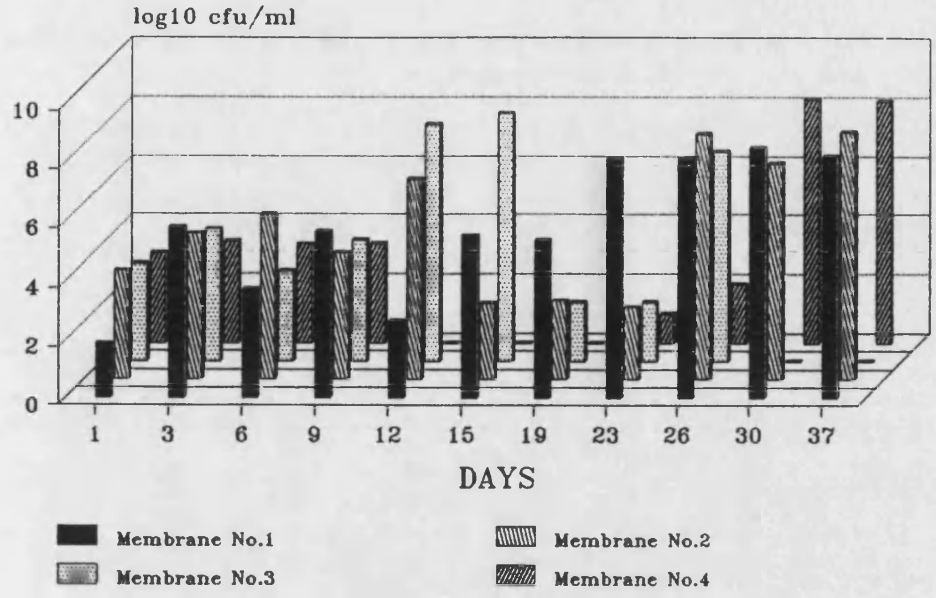
(e) at 4°C. Yolk (Experiment 1)



(f) at 4°C. Yolk (Experiment 2)

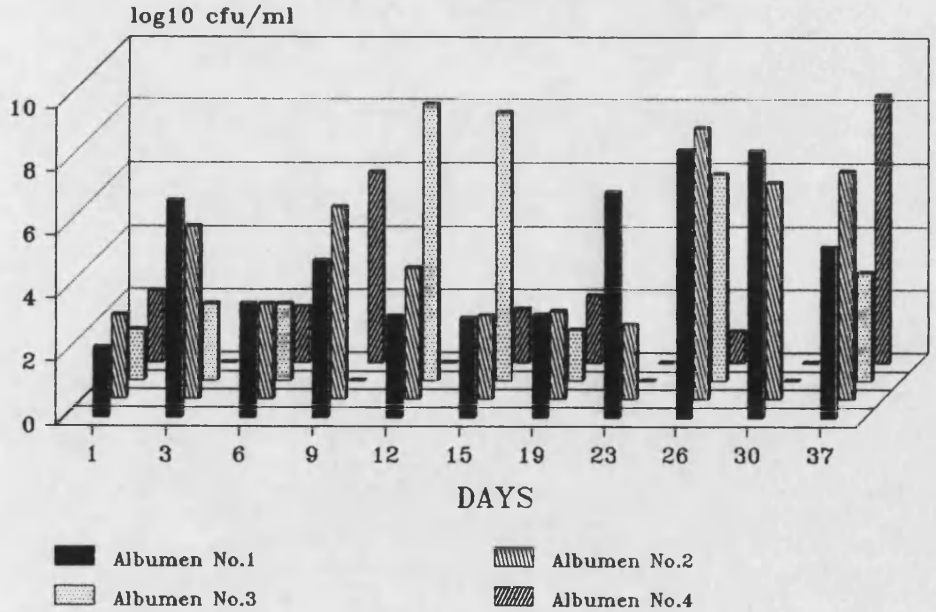


(g) at 20°C. Membrane (Experiment 1)

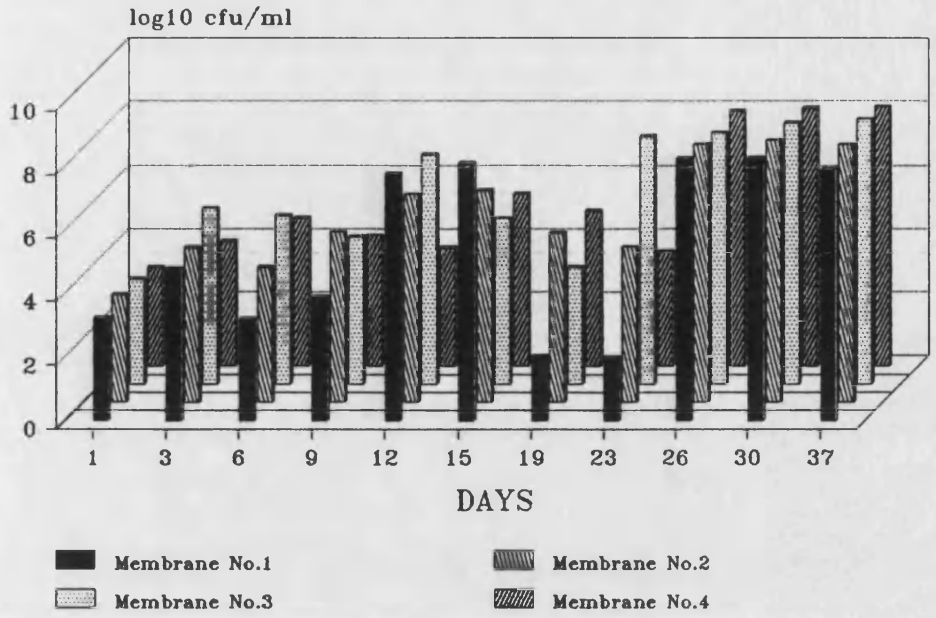


Growth occurred under the air cell membrane on every sampling occasion

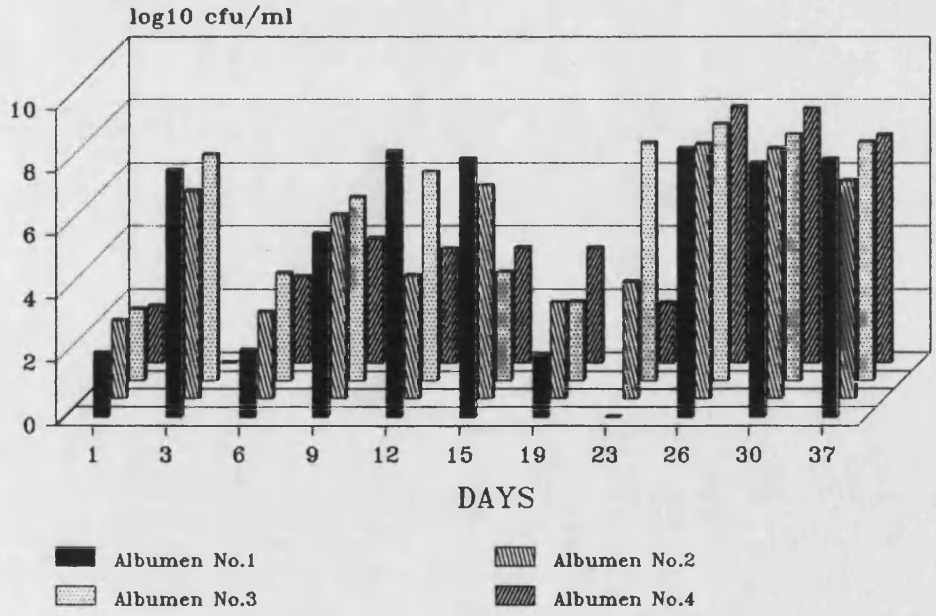
(h) at 20° C. Albumen (Experiment 1)



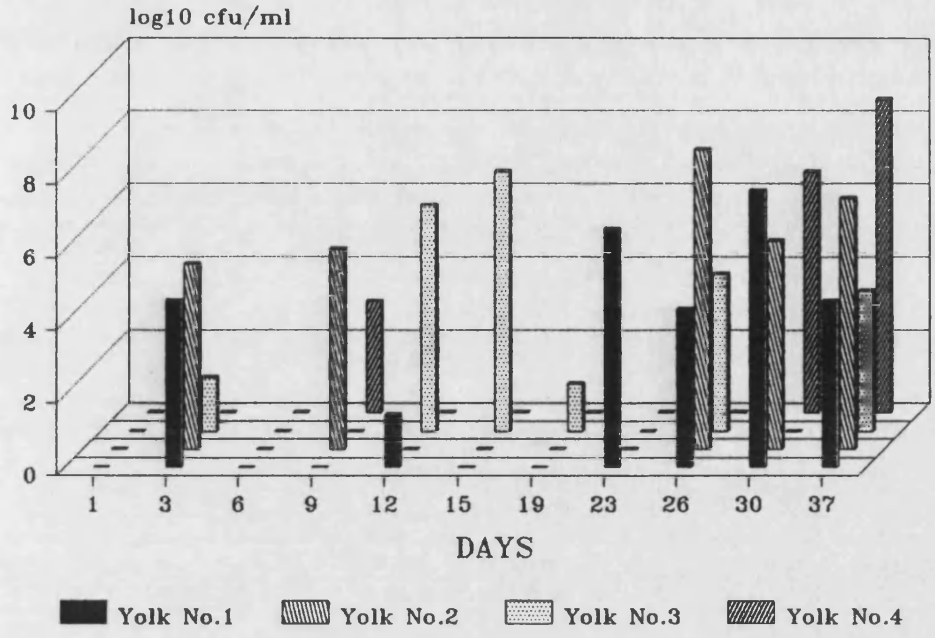
(i) at 20 °C. Membrane (Experiment 2)



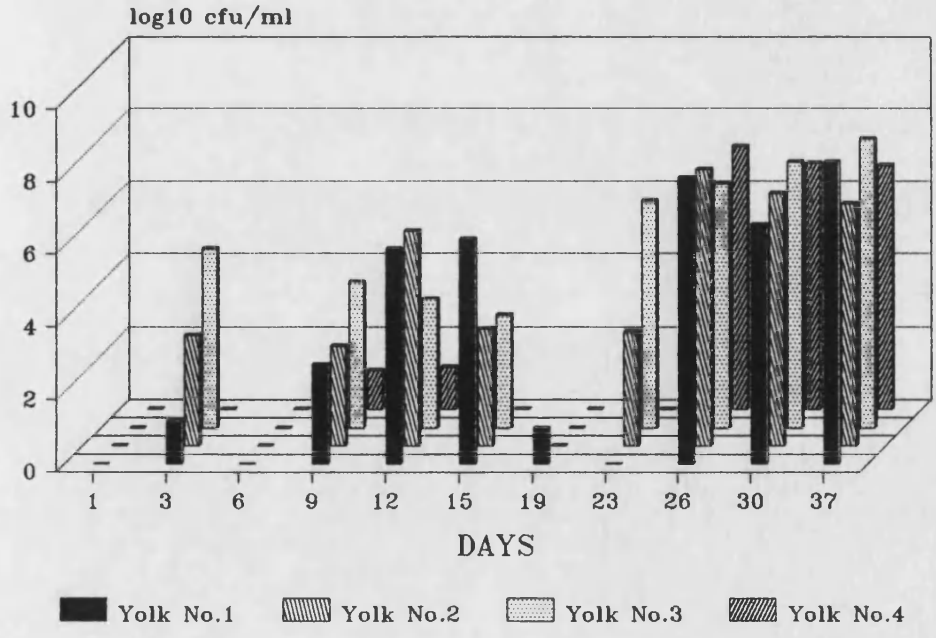
(j) at 20 °C. Albumen (Experiment 2)



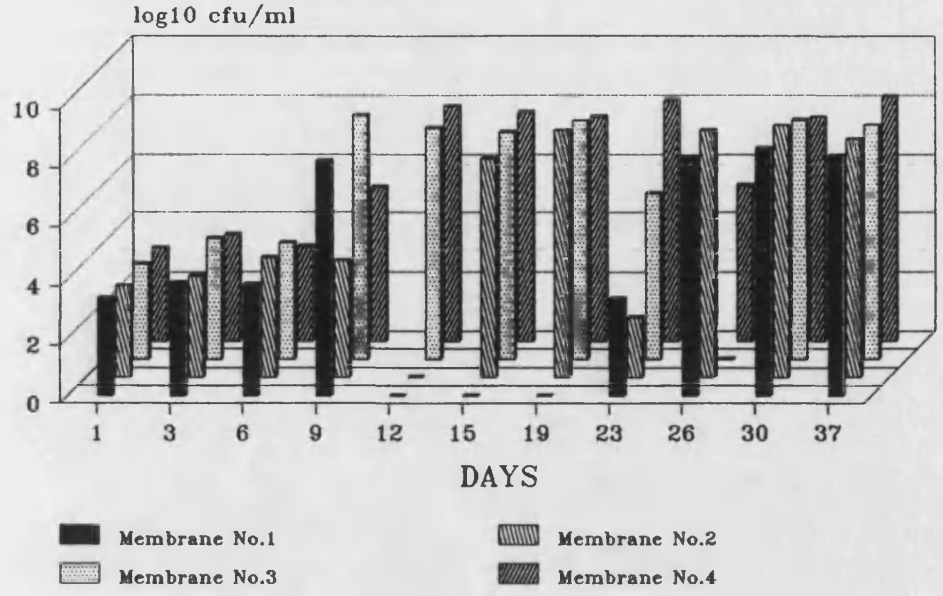
(k) at 20° C. Yolk (Experiment 1)



(l) at 20 °C. Yolk (Experiment 2)

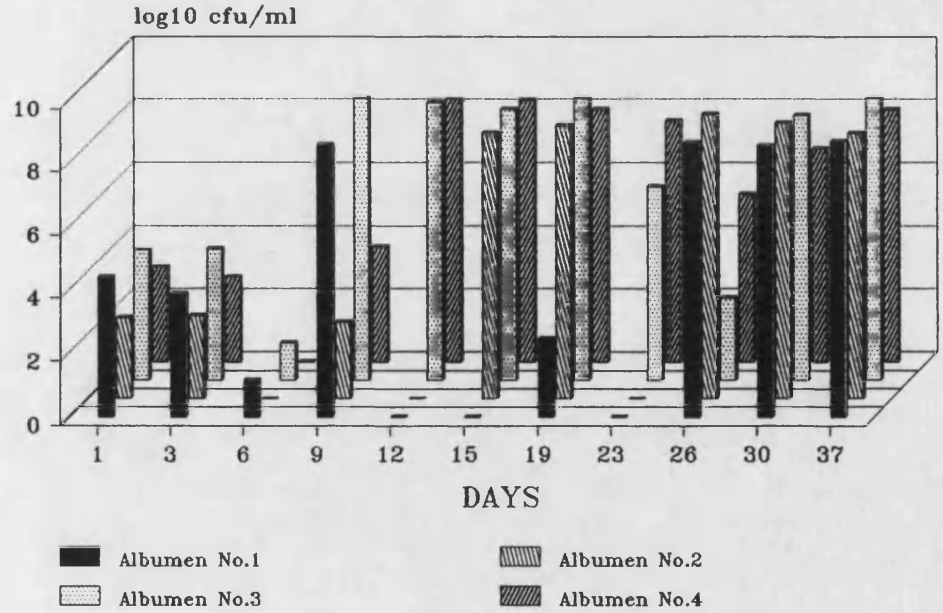


(m) at 30°C. Membrane (Experiment 1)

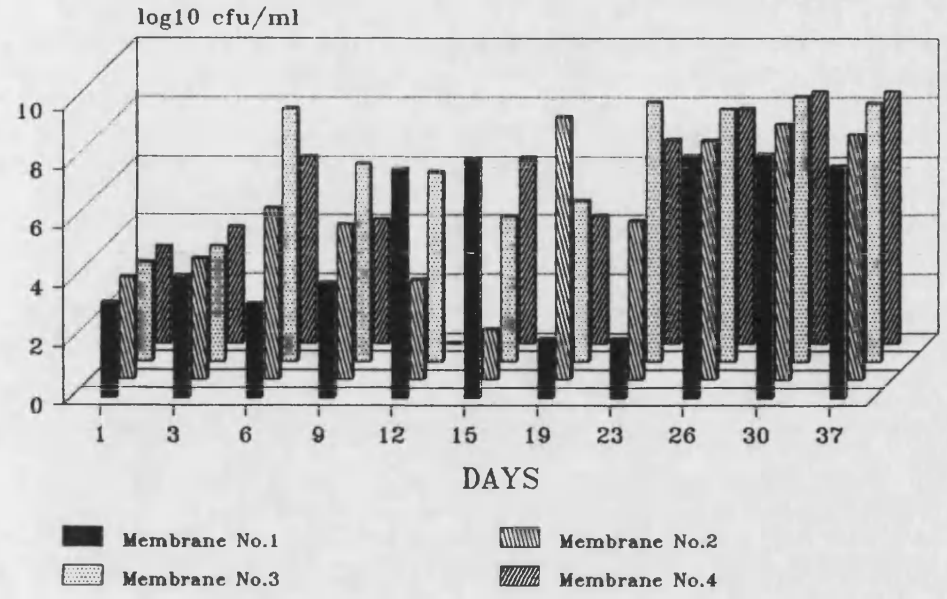


Growth occurred under the air cell membrane on every sampling occasion

(n) at 30° C. Albumen (Experiment 1)

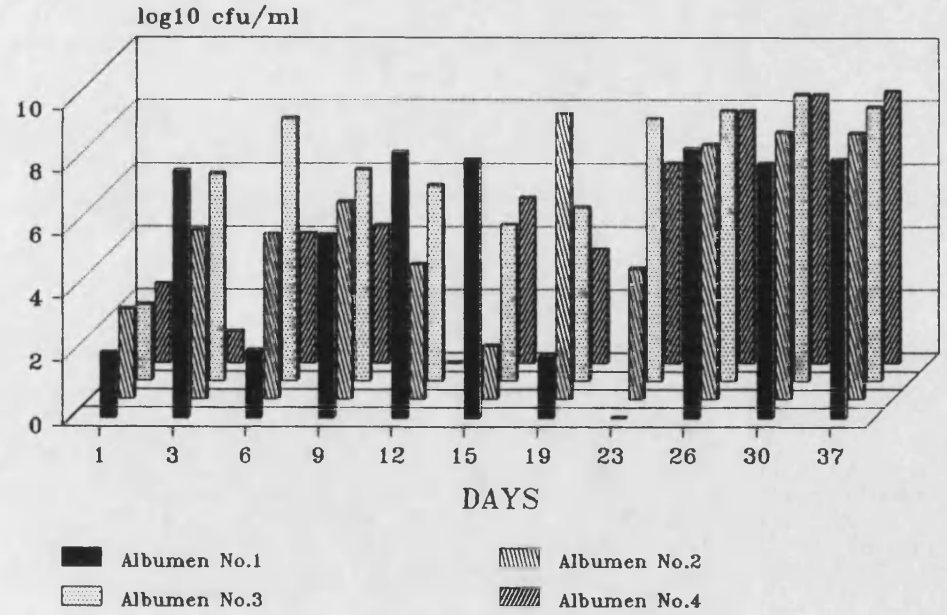


(o) at 30°C. Membrane (Experiment 2)

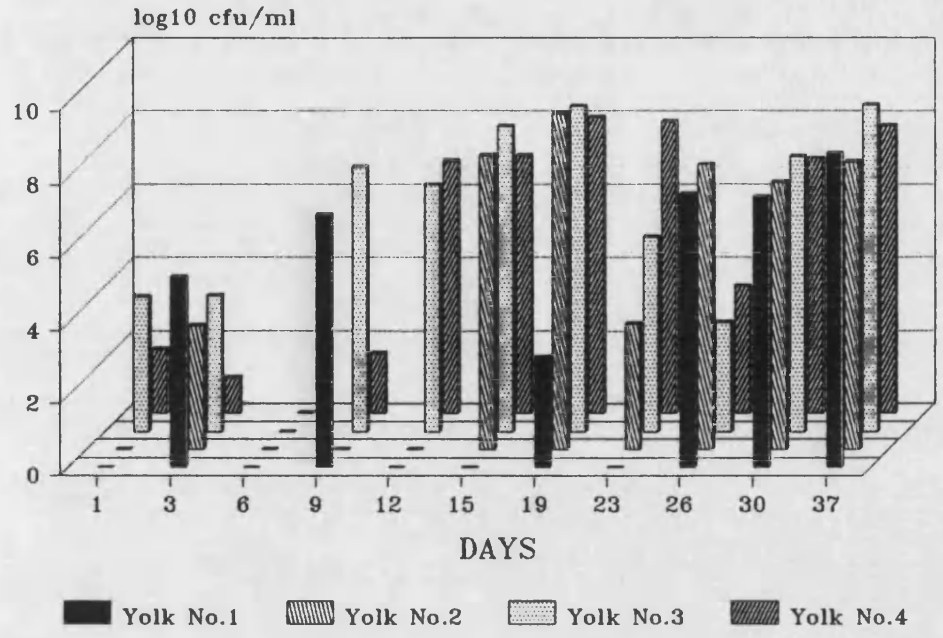


Growth occurred under the air cell membrane on every sampling occasion

(p) at 30°C. Albumen (Experiment 2)



(q) at 30°C. Yolk (Experiment 1)



(r) at 30°C. Yolk (Experiment 2)

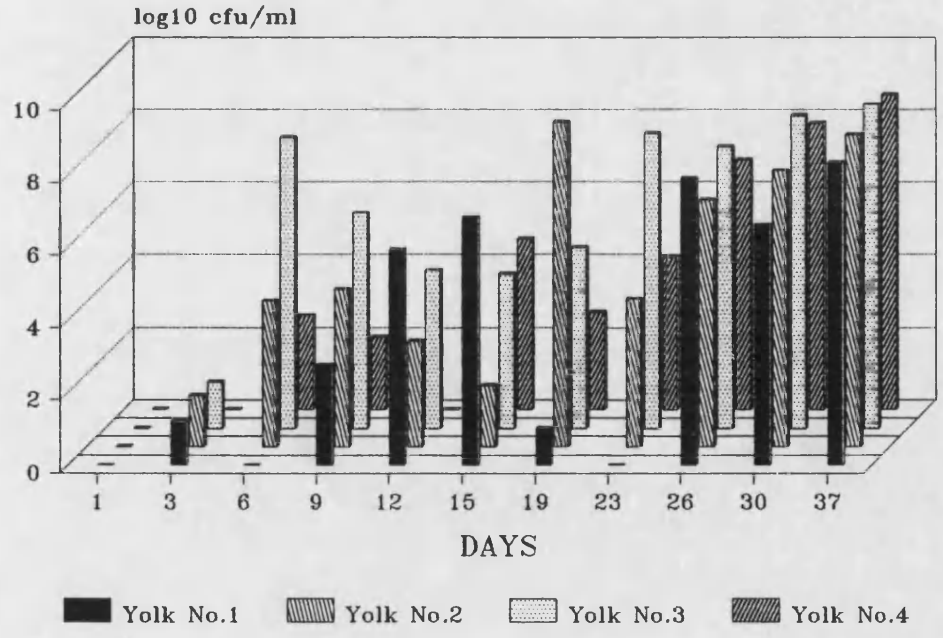
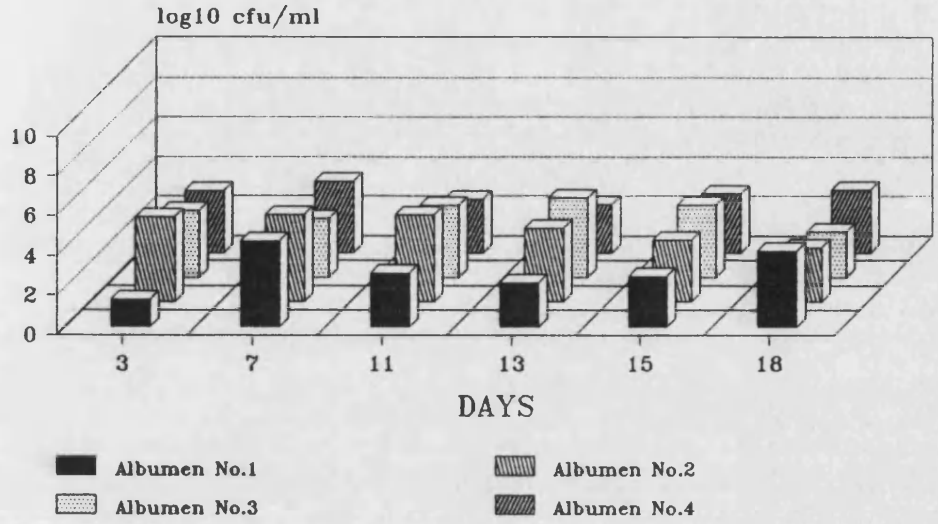
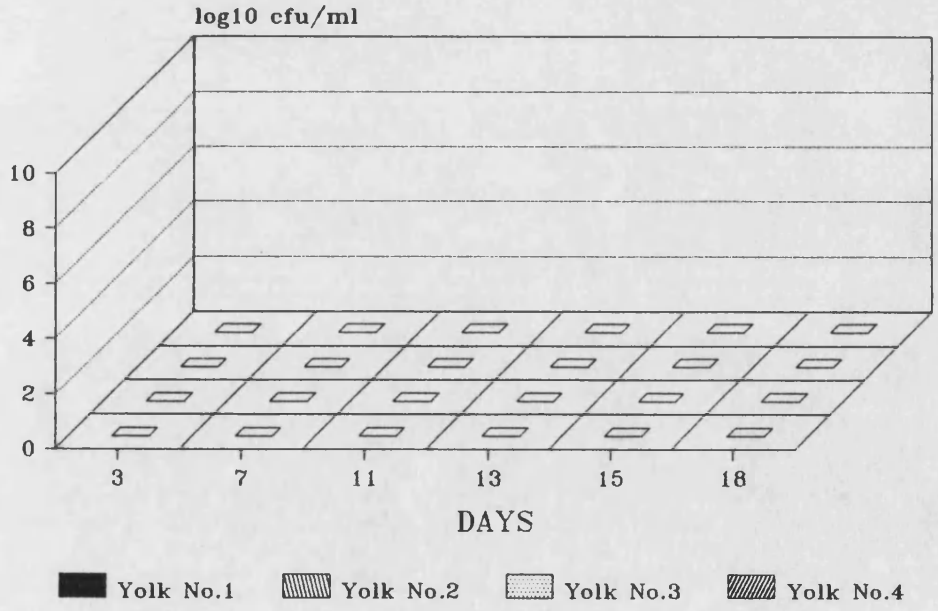


Figure 36 The fate of *S.enteritidis* in whole egg *in vitro*

(a) at 4°C. Albumen (Experiment 1)

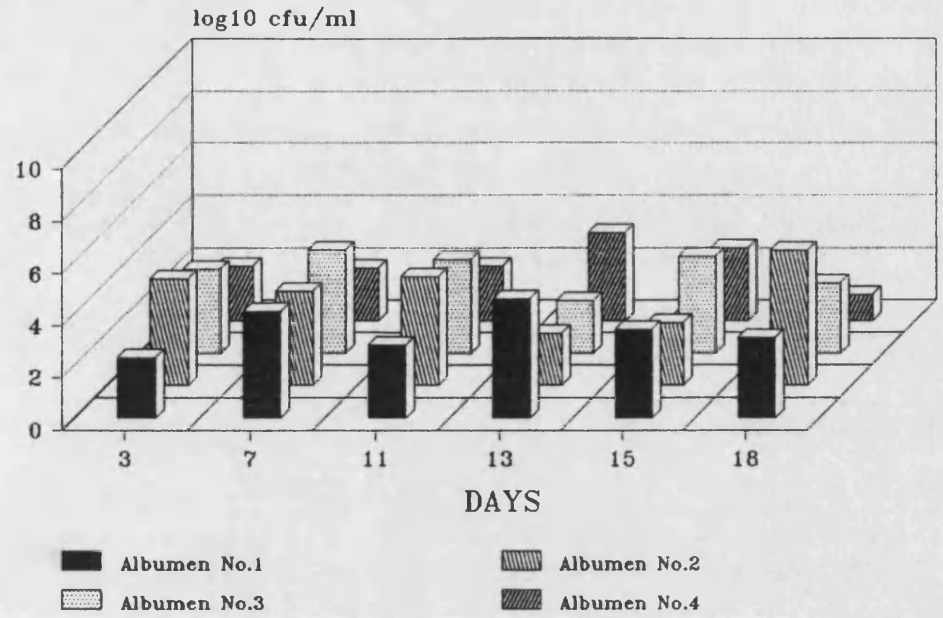


(b) at 4°C. Yolk (Experiment 1)

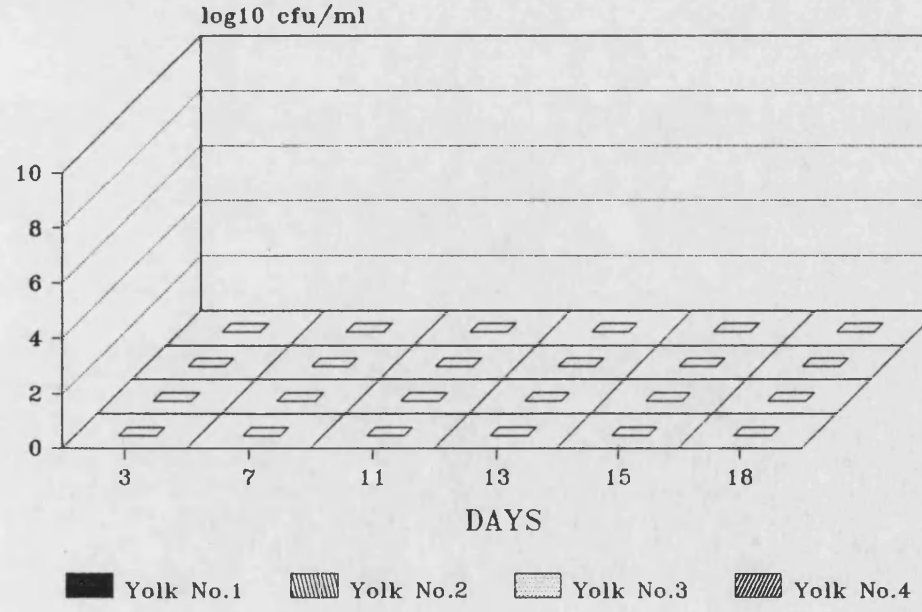


Each seeded plug contained ca. 27,000,000/plug

(c) at 4°C. Albumen (Experiment 2)

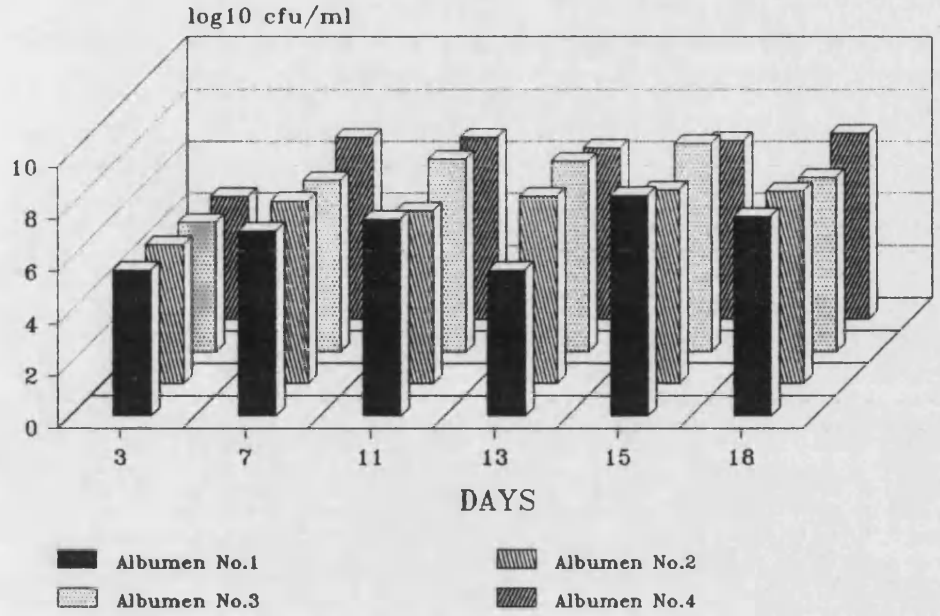


(d) at 4°C. Yolk (Experiment 2)

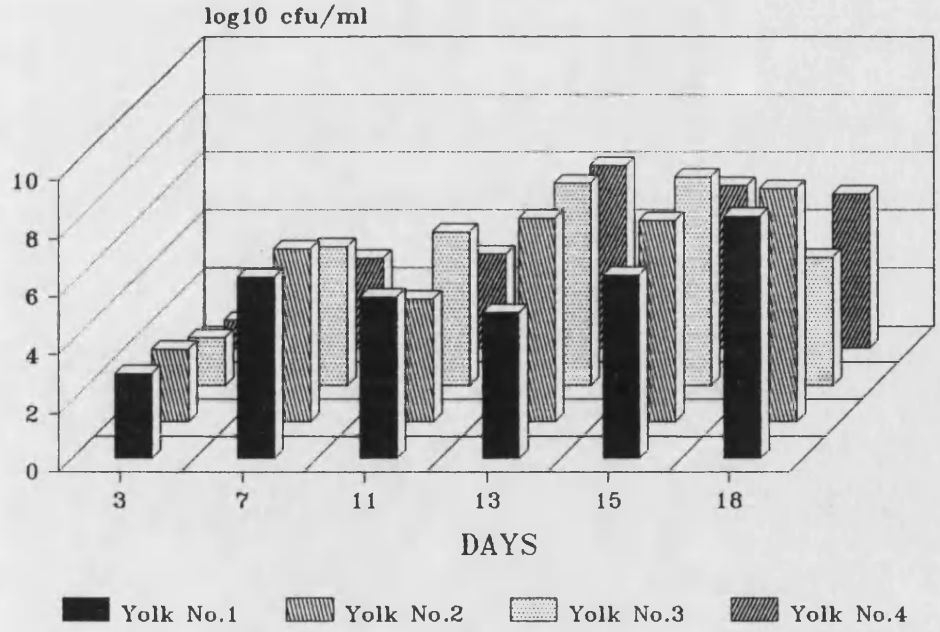


Each seeded plug contained ca. 3.5,000,000/plug

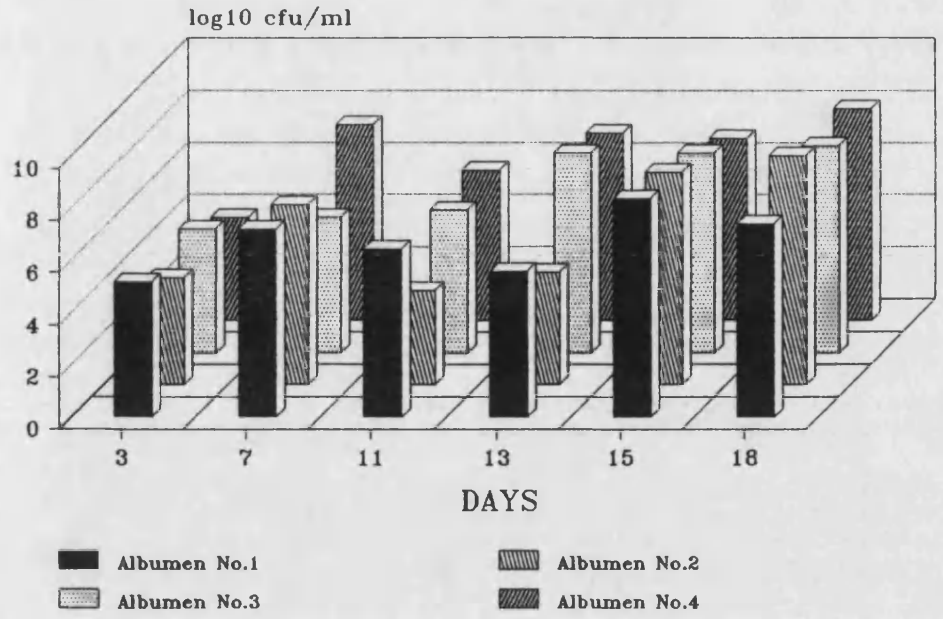
(e) at 20°C. Albumen (Experiment 1)



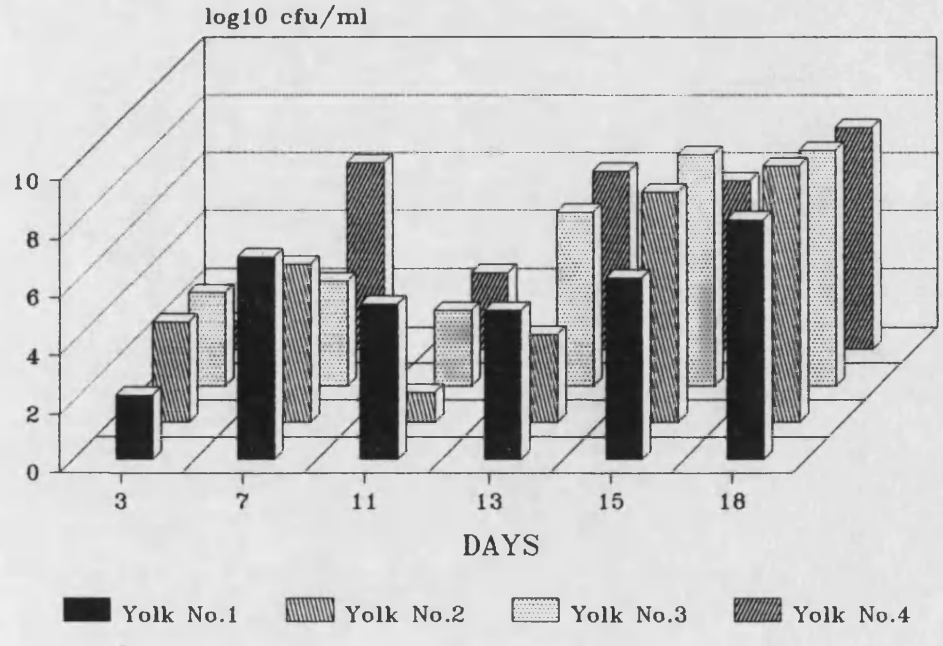
(f) at 20°C. Yolk (Experiment 1)



(g) at 20°C. Albumen (Experiment 2)

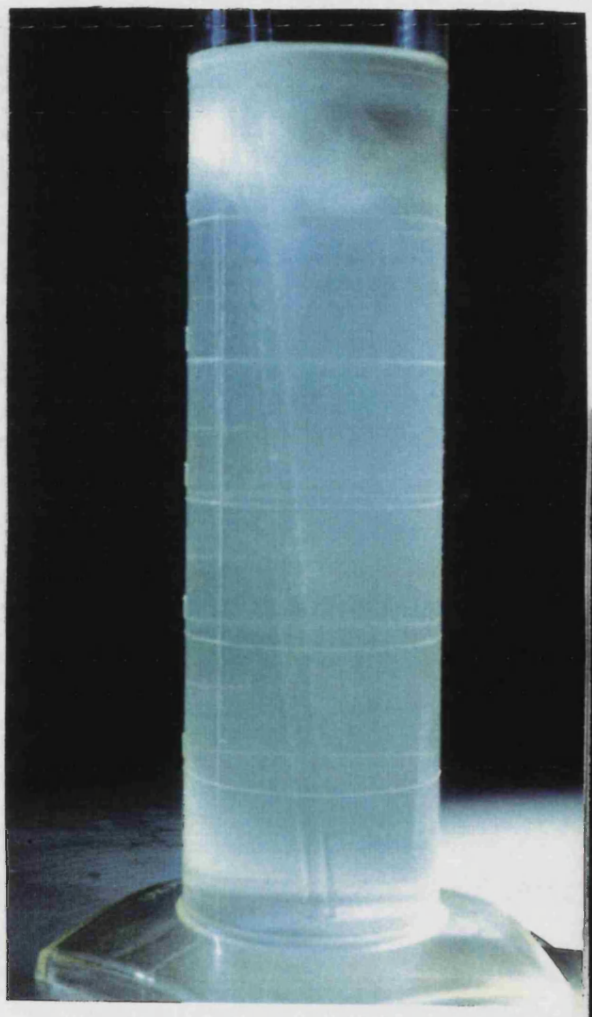


(h) at 20°C. Yolk (Experiment 2)



A method to study the possible role of chemotaxis of *Salmonella enteritidis* induced by the egg yolk *in vitro*

Plate 1. A dense cloud of organisms around the yolk



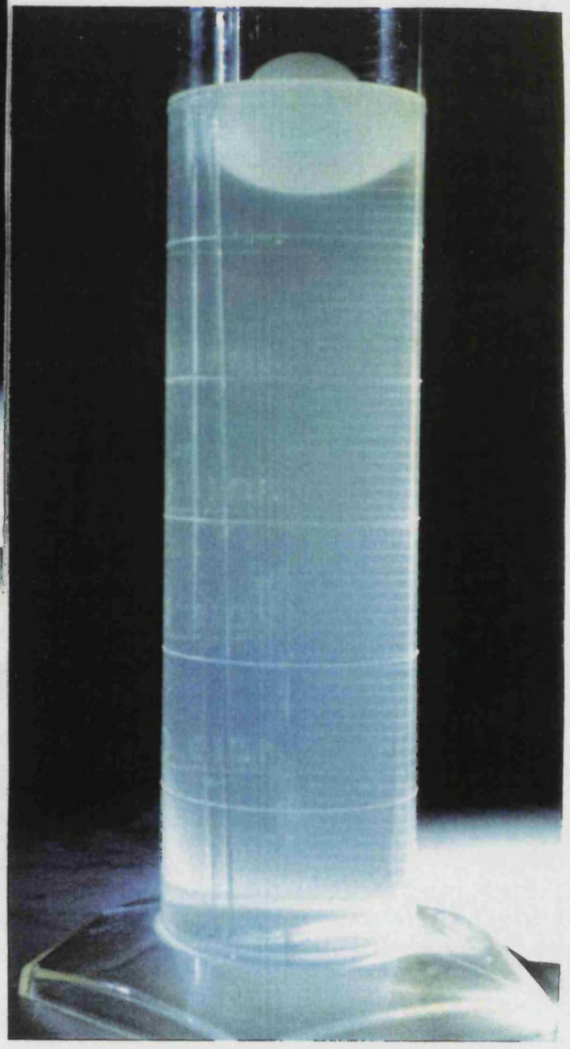


Plate 2.

There was no cloud formation in the presence of a control (ping-pong ball)

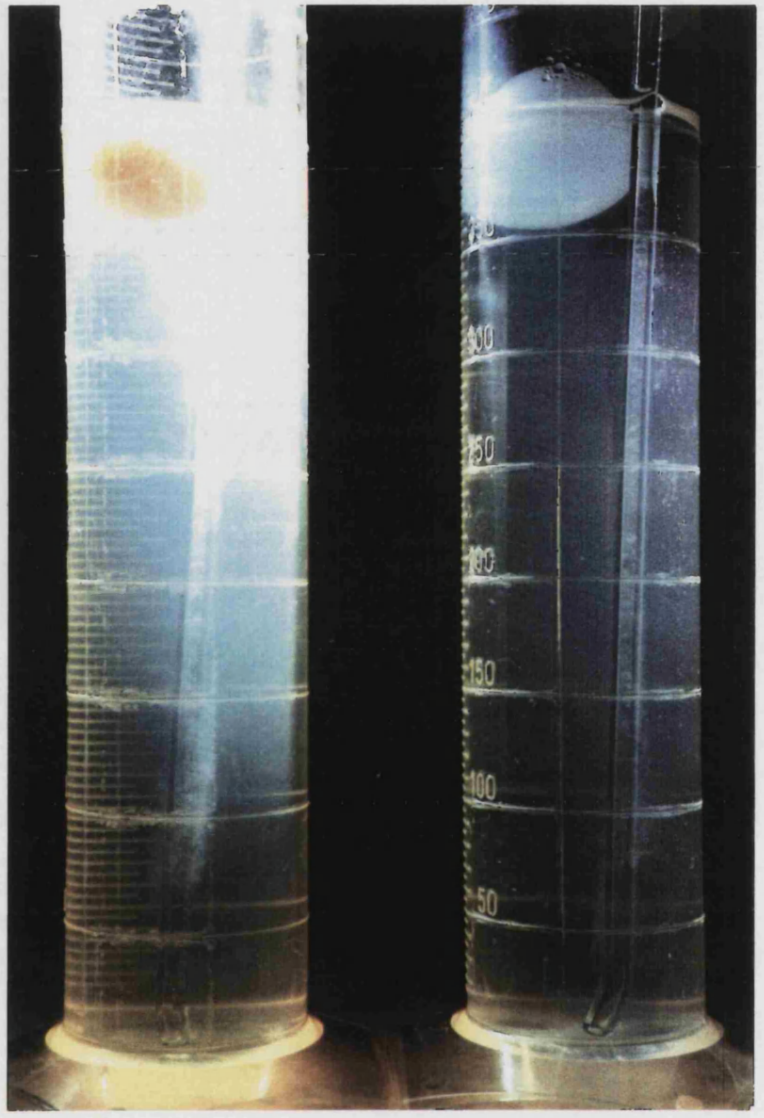
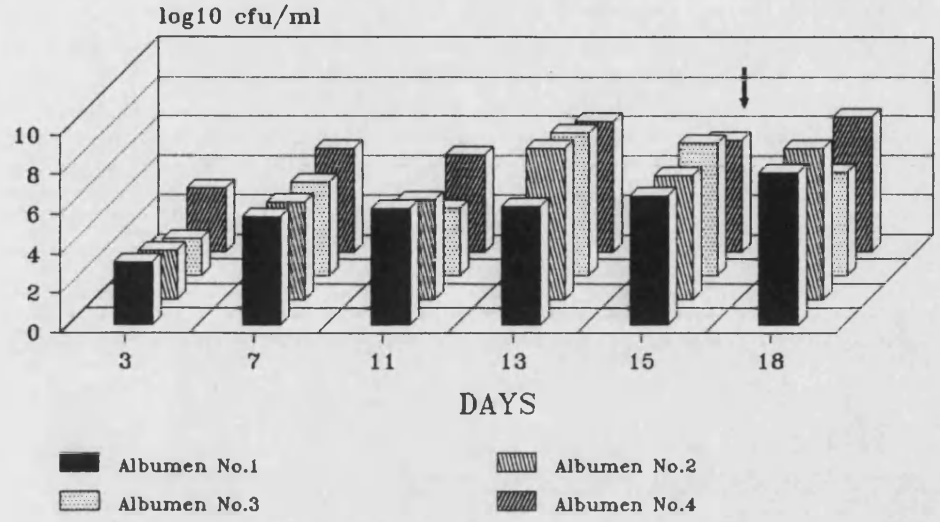


Plate 3. A repeat experiment with the same changes as noted in Plates 1 and 2

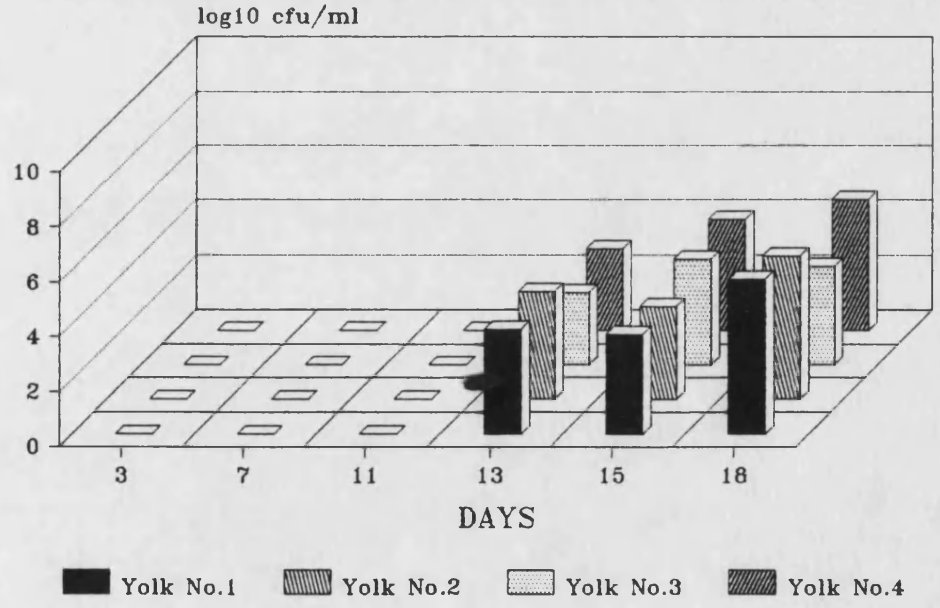
Figure 37 The fate of *Ps.putida* in whole egg *in vitro*

(a) at 4°C. Albumen (Experiment 1)



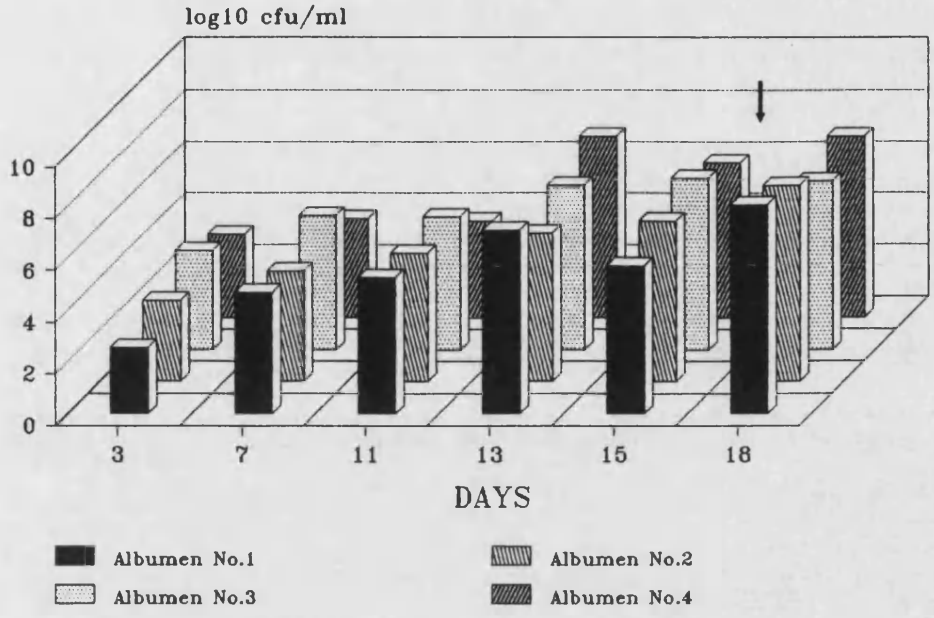
Onset of fluorescence ↓

(b) at 4°C. Yolk (Experiment 1)



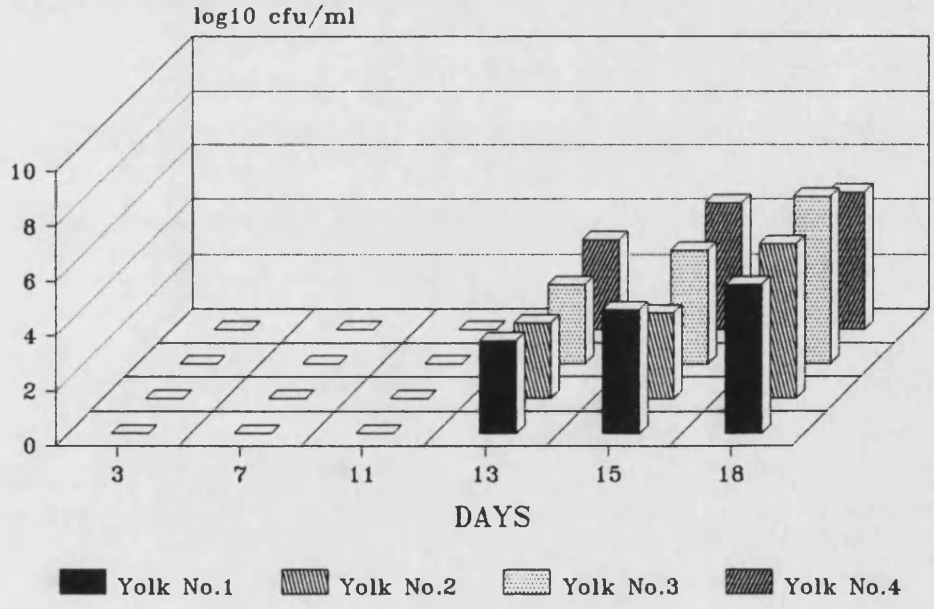
Each seeded plug contains ca. 8,100,000/plug

(c) at 4°C. Albumen (Experiment 2)



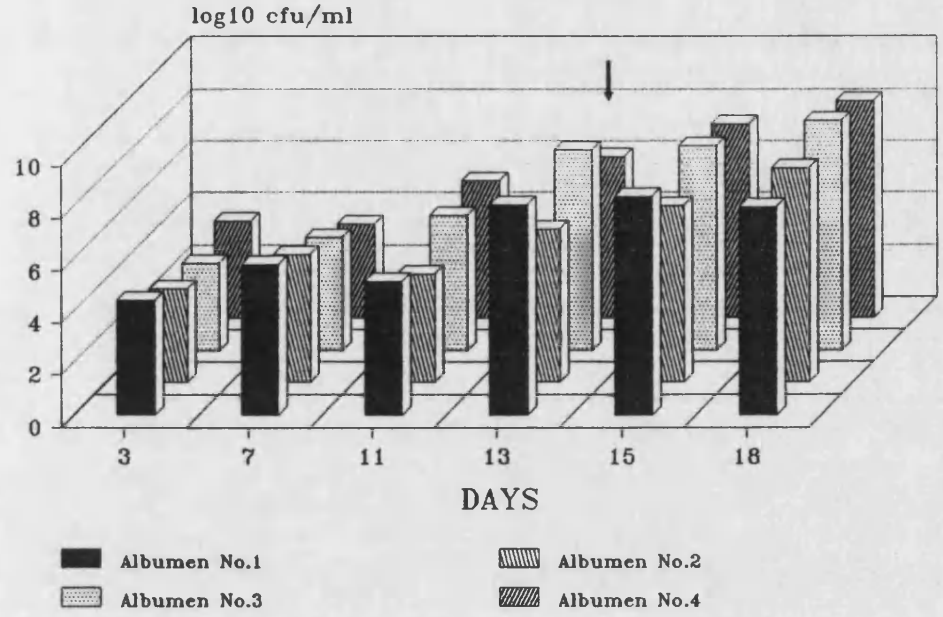
Onset of fluorescence ↓

(d) at 4°C. Yolk (Experiment 2)



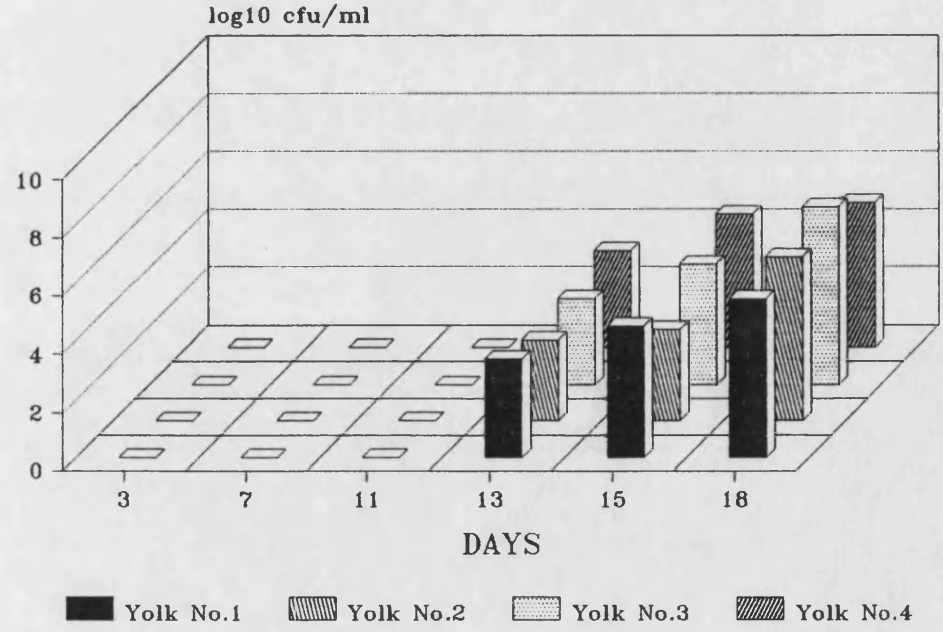
Each seeded plug contains ca.
9,600,000/plug

(e) at 20°C. Albumen (Experiment 1)

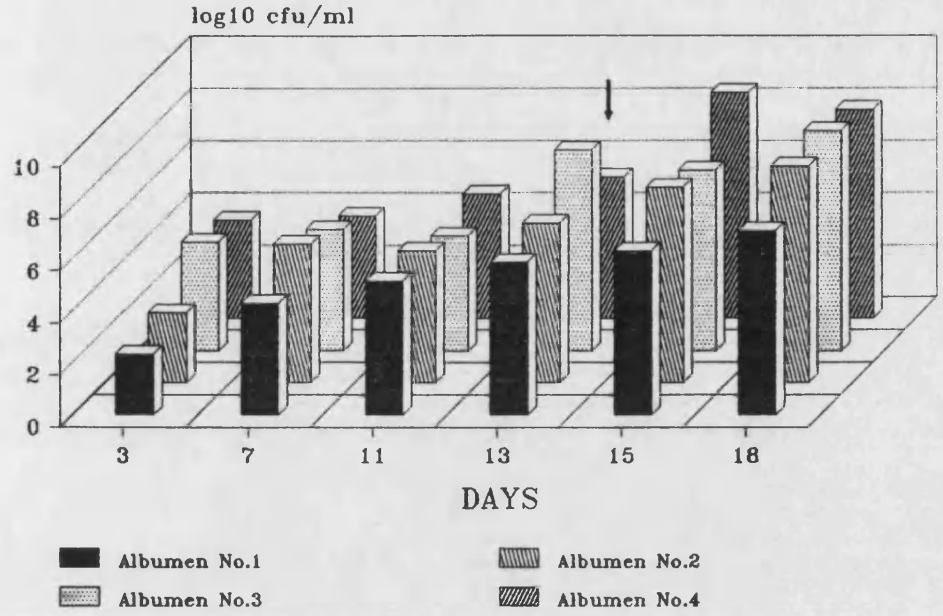


Onset of fluorescence ↓

(f) at 20°C. Yolk (Experiment 1)

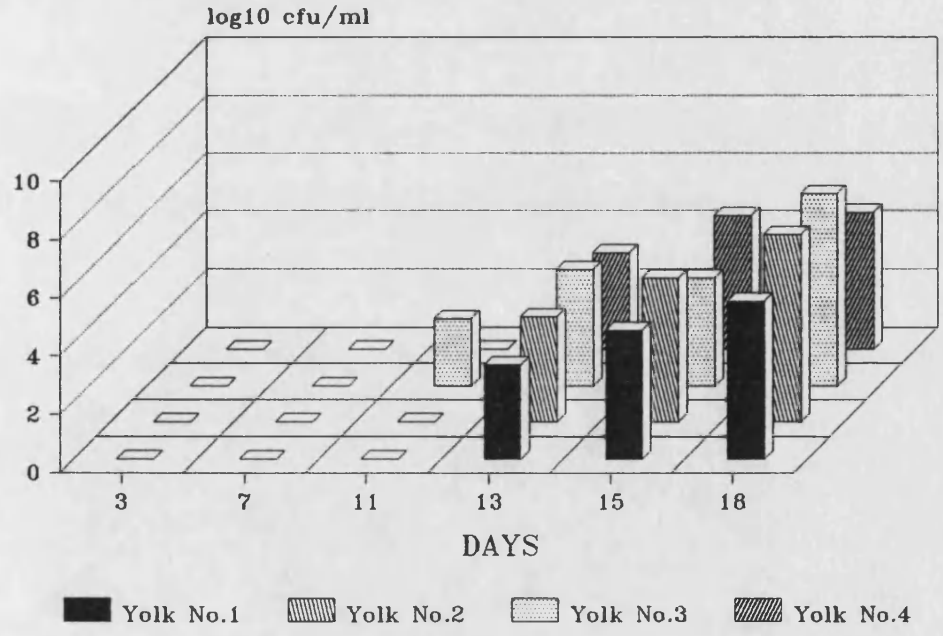


(g) at 20°C. Albumen (Experiment 2)



Onset of fluorescence ↓

(h) at 20°C. Yolk (Experiment 2)



The course of infection- *Pseudomonas putida* in whole fresh eggs at 4 ^{0}C

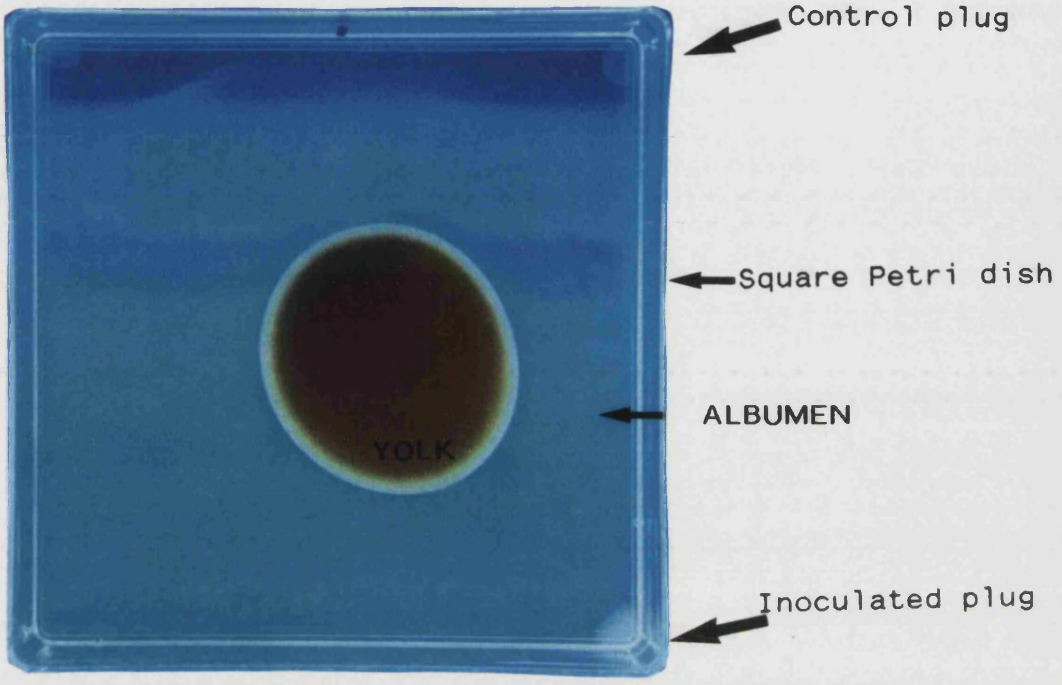


Plate 4. Structure and experimental design are evident from this photograph

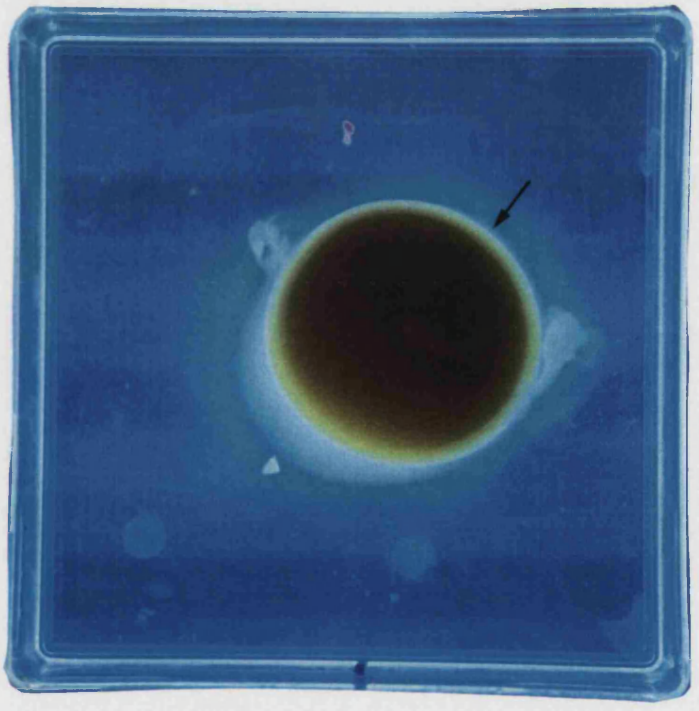


Plate 5. Day 16. A halo of fluorescence begins to develop around the yolk

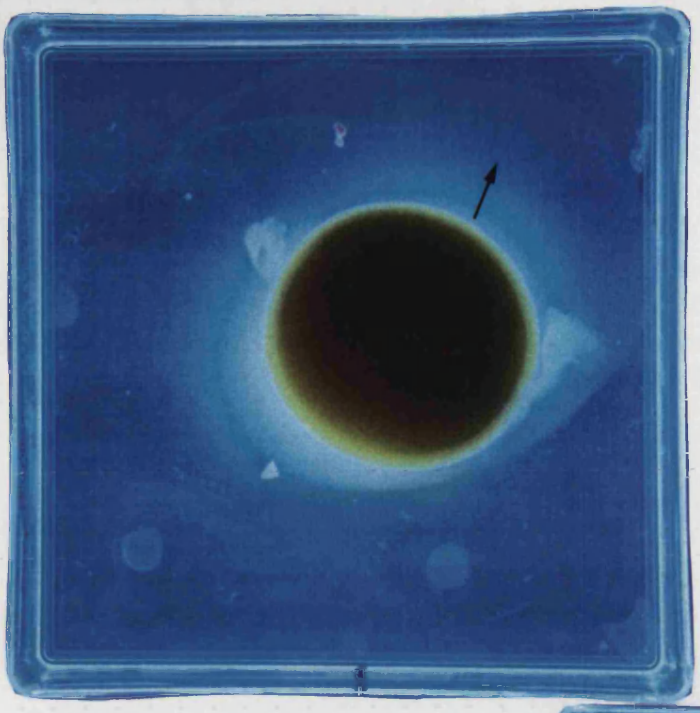


Plate 6.

Day 29. The organism spreads outwards from the thin white enveloping the yolk

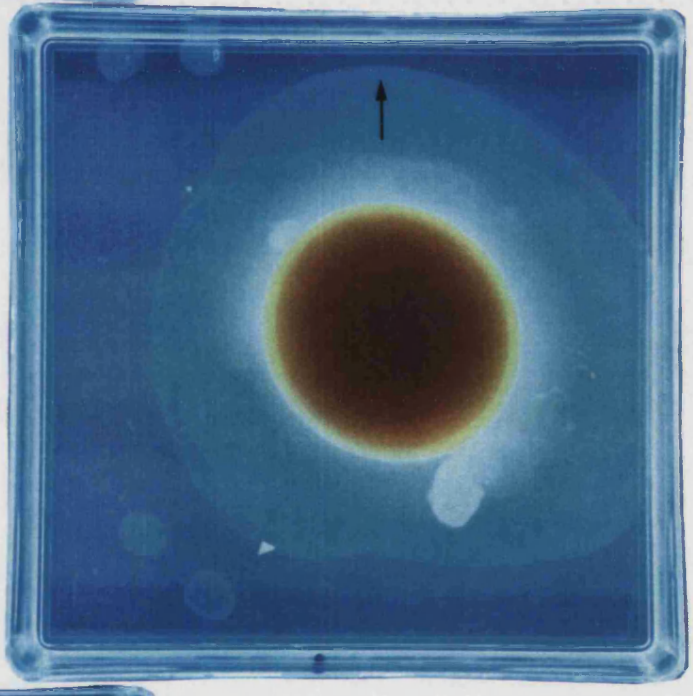


Plate 7.

Day 33. The organism spreads outwards almost to the boundaries of the albuminous sac

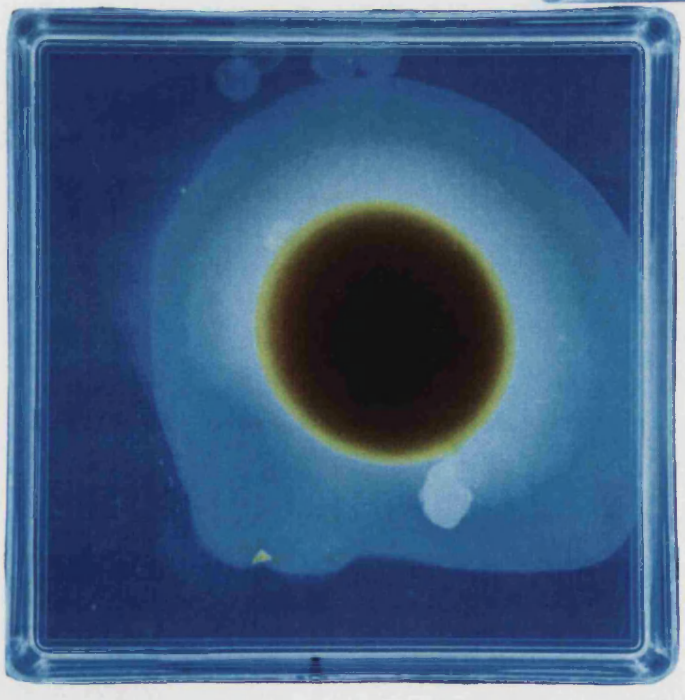


Plate 8.

Day 50. The albuminous sac appears to be saturated with organisms

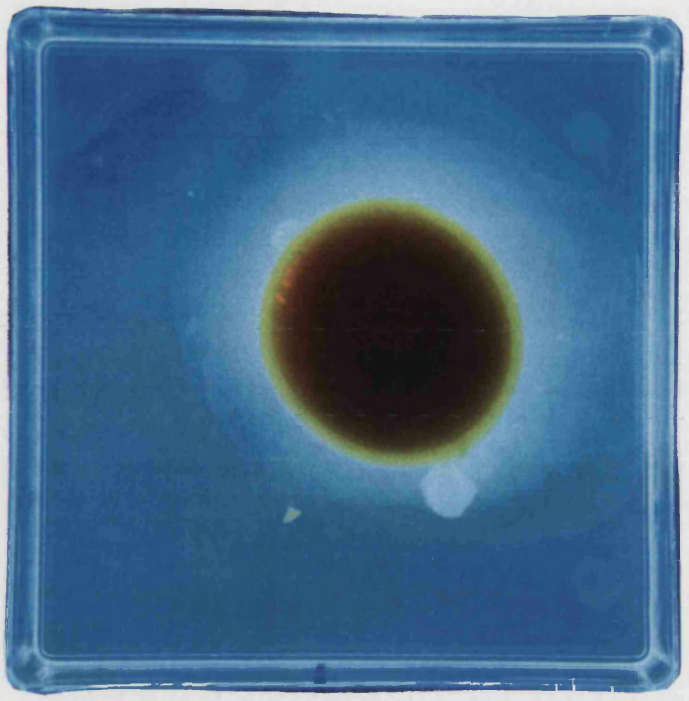


Plate 9.

Day 58. The organisms migrate from the albuminous sac and cause slight clouding of the thin albumen

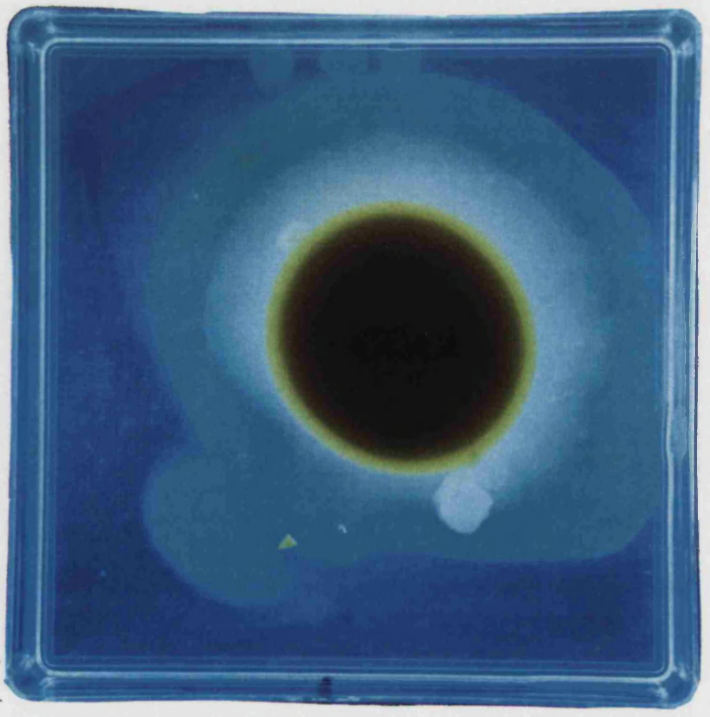


Plate 10.

Day 64. Clouding of the thin albumen is accentuated

The course of infection- *Pseudomonas putida* in whole fresh eggs at 20 ^{0}C

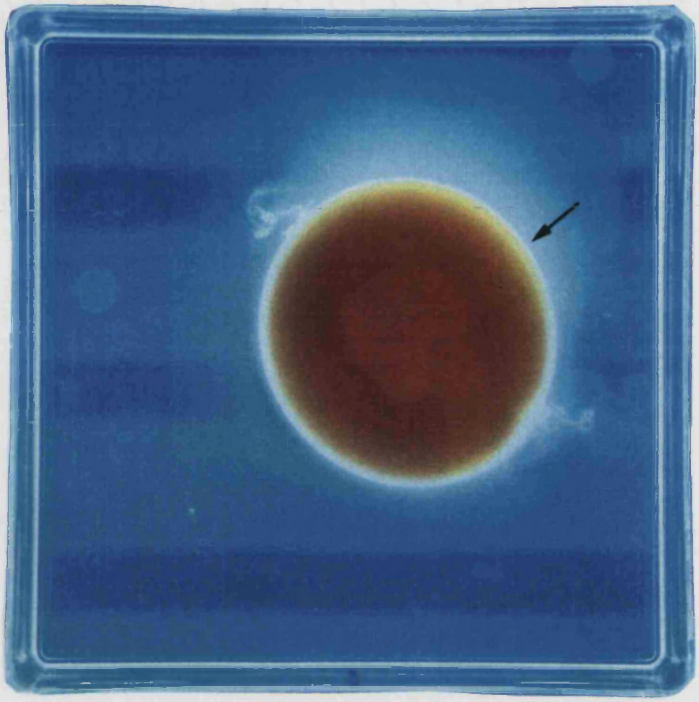


Plate 11. Day 13. A fluorescent halo begins to develop in the thin albumen enveloping the yolk

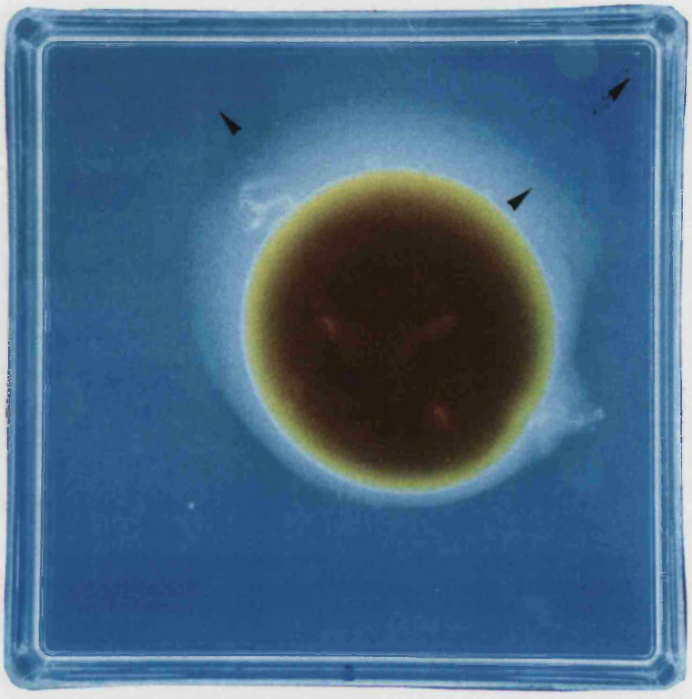


Plate 12. Day 22. The organism spreads into the outer thin albumen

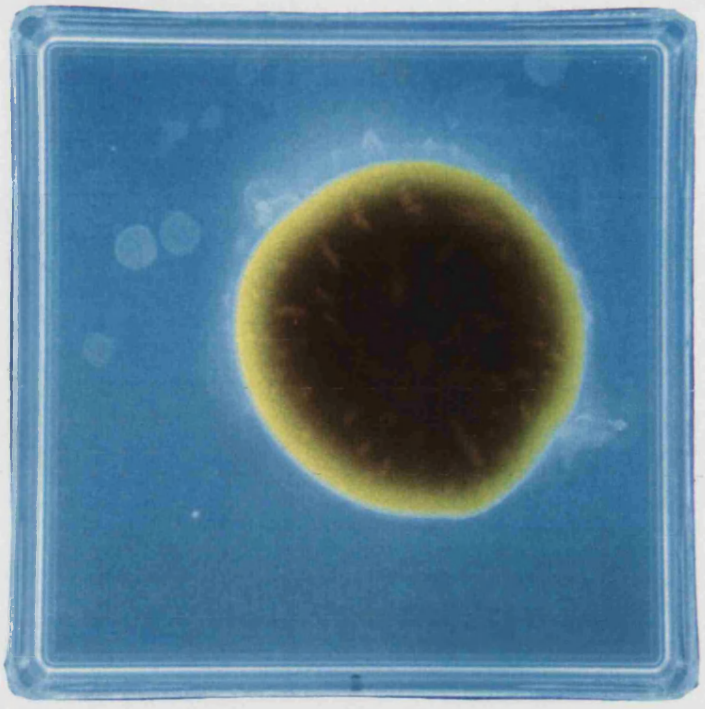


Plate 13. Day 36. The whole egg is fluorescent

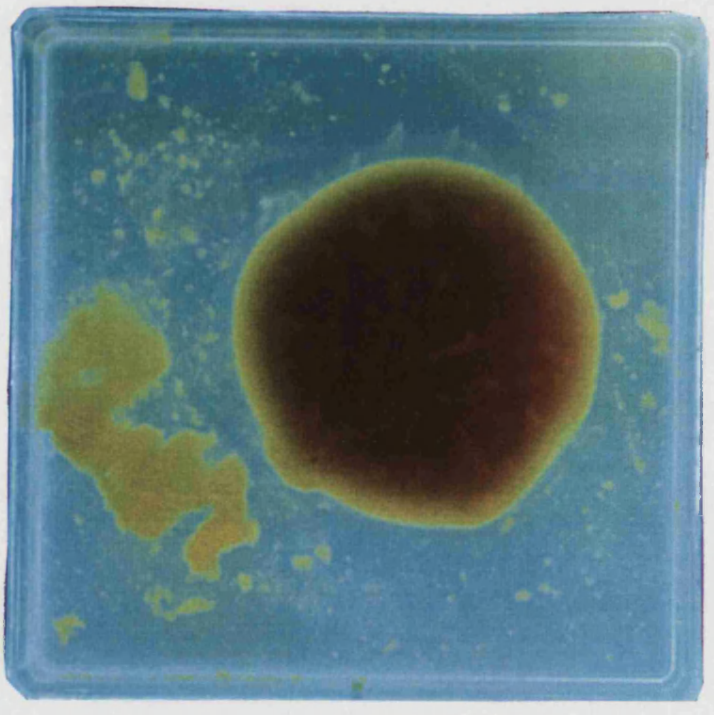


Plate 14. Day 44. Complete breakdown of the egg structure and the vitelline membrane has ruptured

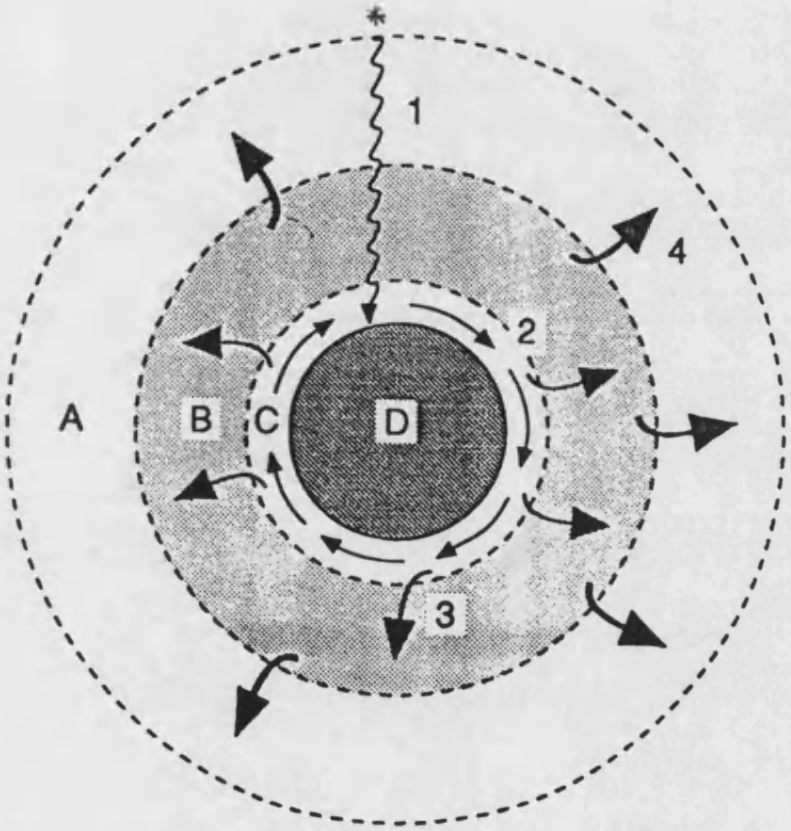


Figure 38. Schematic representation of the course of bacterial infection of the hens' egg

A. Outer thin albumen
B. Albuminous sac
C. Inner thin albumen
D. Yolk

(1). Organisms present in the albumen (or the shell membranes)* persist until there is either a chance, or more probably a chemotactically induced collision with the yolk

(2). The organisms are confined initially to the thin white surrounding the yolk

(3) With time the organism spreads outwards into the albuminous sac

(4). Gross infection of the entire egg contents

DISCUSSION

Even though the pandemic of egg-associated salmonellosis in humans has been with us for upwards of a decade, there still appears to be a notable lack of a coherent view that could be expected to lead to pertinent control measures. Thus as recently as 1992 Gast and Beard have stated that "epidemiological evidence indicates that egg-associated human *S.enteritidis* outbreaks are generally the result of a series of 3 independent events". These are that *S.enteritidis* infected eggs must be produced by hens which are themselves infected. These eggs must then be subjected to an inappropriate food handlng practice that allows the contaminating organisms to multiply. Lastly the food product must be undercooked or consumed raw. These authors recommend therefore that the following steps be taken to prevent infection *via* contaminated eggs. Firstly to adopt refrigerated storage of eggs - at present in the USA Grade A eggs must be stored at 8 ^{0}C or above, and secondly that good kitchen practices are followed eg. avoidance of holding food (especially composite ones) at warm temperatures and ensure adequate cooking.

There has been much discussion about the relative importance of three possible routes of infection of the egg contents *viz* transovarian, oviducal or trans-shell (Duguid and North 1991). As was evident in the Literature Review many attempts have been made to clarify this key issue. The results to date indicate that the

size of the infective dose and the route of infection of the hen, as well as the mode of infection of its egg influences the actual incidence of egg infection. Support for this view comes from the study of hens deliberately inoculated with large or small doses of *S.enteritidis*.

Hens inoculated orally with ca. 10^9 cells of PT 13a led to 3% contamination of the melanged egg contents (Gast and Beard 1992). Bale and Hinton (1991 - unpublished) dosed laying hens at 50 weeks of age orally with ca. 10^9 cfu/ml, or cloacally with ca. 10^8 cfu/ml. A significantly larger number of contaminated eggs were laid by hens inoculated cloacally compared to those dosed orally (9.15% and 3.78% respectively). Despite the large number of positive (melanged) eggs detected during the initial examination, the latter investigators were able to isolate the organism from the albumen of only one egg (8 cfu/g albumen) after a more detailed study of the albumen and yolk. None of the yolks was found to be positive. These authors obtained similar results when the organism (10^6 to 10^7 cells) was given orally to 19 week old pullets. The albumen of 0.27% eggs was found to be positive (<10/ml). These observations suggested that eggs were infected as they travelled from the ovary down the oviduct. This contention has received support from Shivaprasad *et al.* (1990) who found that the albumen was more frequently contaminated than the yolk following oral, intravenous or intracloacal inoculation of hens.

Indeed they concluded that the oviduct or peritoneal cavity was the source of egg contamination.

Less work has been done with smaller inocula. Shivaprasad *et al.* (1990) inoculated hens orally with a human isolate of *S.enteritidis* (10^4/bird). They failed to detect this isolate in any of the samples of albumen or yolk, but they did isolate it from 8-10 egg shells in 3 trials. These authors did, however, detect *S.enteritidis* (an isolate from chicken egg yolk) in 2.7% of albumen samples from eggs produced by birds receiving the same size inoculum (10^4/bird). This isolate was never detected in the yolk.

In practice Logitudinally Integrated Safety Assurance (LISA) as discussed recently by Mossel and Struijk (1992) would appear to provide a totally adequate means of assessing hazards and suggesting remedies in the shell egg industry. Two aspects of LISA applied to egg production need to be recognised. One is the control of infection of the bird with *S.enteritidis.* The second is the manner in which eggs are handled such that they do not pose a risk to man. The various features of the first mentioned were discussed in the Literature Review. In this section, a summary will suffice (Figure 39). It is noteworthy that all the control features noted in this Figure have been adopted in the siting and design of a very large breeder unit in the USA (Anon 1992a). The company sought a site offering a low summer temperature and isolated by many miles from adjacent poultry or livestock farms. The housing was custom built to a high

standard and serviced with high quality water supplies and pasteurized feed taken from bins off-site. Additionally all raw materials and feed mill facilities are tested regularly for freedom from salmonellas. A comprehensive programme of serological testing of birds is also carried out. In addition all dead-on-arrival chicks and first week mortalities are sent to a laboratory for examination. A cloacal swab of every bird is screened every 3 weeks, litter is tested and bacteriological media are monitored for their ability to support the growth of *Salmonella* spp.

Recently egg producers in Holland have been introduced to the "automatic nest" (Anon 1992b). The nest base is formed from rubber fingers that allow air to circulate, thereby ensuring rapid cooling and drying of eggs. The fingers also prevent the eggs rolling around in the nest thereby minimizing shell cracks and damage to the cuticle. The base of each nest is easily removed and disinfected if heavy soiling occurs. The eggs are removed when the base rises gently in the nest, at a preset time in the laying cycle. This serves also to remove debris from the eggshells as they roll over the fingers of the nest base. On removal the eggs roll into square holes in a conveyor belt. This again prevents the egg from rolling and protects them from micro-cracks in the shells. If an egg should break in the conveyor belt, the contents do not contaminate other eggs but drip through the holes in the conveyor. This type of regime could well become standard in developed countries as

commercial pressures force small breeders/egg producers out of the industry and the growth of large well managed production units is encouraged by the demands of supermarkets.

Indeed pressure from the latter could well lead to a cold chain being established from the laying house to the consumer. Such a chain already exists with Grade A eggs in the USA with a temperature of 8 ^{0}C being used. This temperature was chosen because it minimizes "sweating" - water condensation on the shell - when eggs are transferred to ambient temperature. There is a widely held view that "sweating" could lead to trans-shell infection of eggs. This may have been a problem when many eggs were stored for long periods before being shipped along uncontrolled ditribution systems to the consumer. As far as can be assertained, "sweating" need not be a problem today because it would occur only at the stage immediately before the use of an egg in the home or commercial kitchen. As 8 ^{0}C is outside the range used for other chilled foodstuffs, an inconvenient twin system of chill storage is required in supermarkets. Consequently attempts are being made to adopt the same temperature for eggs and chilled food storage (Bruce *pers comm*). What ever policy is adopted for the transport of eggs from the farm to the consumer, it ought to reflect the findings of this thesis.

With the emphasis given to a "clean" appearance, supermarkets may well insist on the washing of all table eggs. The effect of egg washing must therefore be

discussed. There is no evidence to suggest that eggs correctly washed are a greater source of infection to man than unwashed ones (Moats 1978). Sparks (1992) has shown, however, that washers incorporating rotating brushes accentuate the rate of cuticle deterioration and decrease its ability to resist water uptake by the egg. This combination of events, together with prolonged storage of eggs, may well lead to gross infection of the egg contents. This emphasizes the need for rapid movement of eggs from producers to the consumer. If irregular demand for whole egg products such as scrambled eggs calls for long-term storage of shell eggs, management ought to consider the use of pasteurized products. In the USA these are available in ½ or one pint containers (Bruce *pers comm*). Another problem inherent in egg washing is the abuse of the system by the repeated washing of heavily soiled eggs. There is a much higher risk of contamination of the egg contents with infected wash water, and greater damage to the cuticle if this takes place (Sparks 1992). The practice of repeat washing should therefore be discouraged. Moreover it has been demonstrated in this thesis and by Clay and Board (1992) that faecal extract accentuates the growth of bacteria that contaminate the shell membranes.

From the Literature Review it was evident that the course of infection of an egg contaminated *via* the trans-shell route or oviducally is similar. Indeed the lag of 10-20 days between infection and gross contamination needs to be stressed (Table 6). This present study was

intended to identify more precisely the events which follow initial contamination of the shell membranes or albumen.

All work with eggs inoculated artificially with salmonellas indicated that the course of infection was the same as that in eggs infected with rot producing bacteria (Board 1964). The major difference was that growth of *S.enteritidis* in whole eggs does not change the appearance of the egg contents (Clay and Board 1991). The course of the infection with rot producing bacteria has been studied over many years. The studies have led to the identification of several stages in the infection process (Board and Fuller 1974).

The first of these stages involves the contamination and penetration of the shell. This aspect was not studied in this thesis. The second is the colonization of the shell membranes. Gillespie and Scott (1950) observed a localized spot of pigment at one point of the shell membrane in eggs infected with pseudomonads, and presumed that this was the site of penetration of the shell. Board and Fuller (1974) state that organisms persist on the shell membrane until some event induces their growth. Until recently there was general agreement based on circumstantial evidence coming from the many studies of infection of eggs with rot producing bacteria (Board 1966), that rapid multiplication occurs when organisms acquire essential nutrients on making contact with the surface of the yolk (Board 1964). Contact with the yolk may result from the latter floating upwards in

the albumen towards the air cell and infected shell membranes. This rise is due to the uptake of water by the yolk and the breakdown in structure of the albuminous sac as the egg ages. Alternatively contact may be by "chance" collision of a contaminant with the yolk. The results presented in this study indicate that the latter may be associated with a chemotactic response.

These studies have shown that organisms inoculated onto the air cell membrane remain quiescent and those that invade the albumen do not proliferate in the absence of the yolk (Figure 34). Indeed the study of many serotypes and phage types of *Salmonella* (Table 10) showed that failure to grow in albumen was a feature of the majority of those tested. Gross contamination of the egg contents occurs when the yolk is present (Figure 35). This was demonstrated with the use of excised air cell membranes inoculated with *S.enteritidis* suspended in albumen alone or the albumen of whole eggs, both held in sterile containers. If the membrane floated onto and lodged on the yolk as the albumen aged, the onset of gross contamination was much faster than was the case when the membrane remained at some distance from the yolk. These events are comparable to those occurring in whole eggs in which the yolk rises towards infected shell membranes (Clay and Board 1991).

My results support existing evidence on the antimicrobial properties of ovotransferrin. Ferric ammonium citrate overcame the antimicrobial properties for all 26 *Salmonella* serotypes and phage types used in

this study, providing incubation was at 20 or 30^{0}C. It has been shown repeatedly (see review by Tranter and Board 1982) that the bacteriostatic action of albumen against Gram negative bacteria is negated by the addition of a combined nitrogen and Fe^{3+} source in sufficient concentrations to saturate ovotransferrin. In such experiments the supplements were added before or immediately following inoculation of the albumen. In the present study bacteriostasis was released by supplements added on the 42nd day of incubation.

Gillespie and Scott (1950) observed a small bright green patch in the albumen of eggs infected with pseudomonads. This they attributed to the site of initial growth in albumen of pseudomonads derived from the shell membrane. Samples of albumen remote from this patch were usually sterile. They concluded that from this point of initial growth gross contamination of the egg contents eventually occurred. Gillespie and Scott (1950) observed fluorescent spots in broken out eggs. Such spots were seen in studies in the albumen of whole eggs inoculated with *Ps.putida* (Dolman and Board 1992). In practice they could not deduce the location of the spots in broken out eggs. The results obtained with eggs in square Petri dishes led me to conclude that they were probably located alongside the yolk and that chemotaxis may well have played a role in the movement of bacteria from the initial site of infection to the yolk surface (Plate 5).

In the context of the marketing of table eggs these observations give emphasis to the need for the rapid movement of eggs from the farm to the consumer. Indeed it is evident that there is a 10-day period at ambient temperature during which an egg infected with salmonella is lightly contaminated and therefore of minimal risk to the average consumer. This safe period could be extended by chill storage.

It may be inferred from the results discussed to date that unsupplemented albumen induces a quiescent state in *S.enteritidis.* Moreover the studies with somnicells suggest that even these cells can multiply rapidly once the chelating potential of ovotransferrin is quenched with Fe^{3+}. This latter state in *S.enteritidis* was noted by Rozak, Grimes & Colwell (1984) who worked on water contaminated naturally with this organism.

It is important to realize that organisms in a hen house, a feed mill or even within a hen may well be in an unfavourable environment compared with the ideal laboratory conditions in which most cultures are maintained before being used in experiments with eggs. I have shown that organisms in the somnicells state may remain viable but non-culturable for upto 42 days in albumen. This state persists until the addition of nutrients or, perhaps, contact of organisms with the yolk induces multiplication. If infection of eggs with somnicells of *Salmonella* were to occur in the poultry industry, then the utility of assessing the status of

laying hens by sampling eggs for *Salmonella* would be a questionable approach.

The results demonstrated that *S.enteritidis* remained motile in albumen for an appreciable period of time (Table 11), this provides evidence of the potential of chemotaxis to influence egg infection. If a chemotactic response is involved, it may be induced by substances diffusing from the yolk. Humphrey *et al.* (1991) have shown that salmonellas inoculated at the periphery of the albumen or at the mid point between the edge of the albumen and yolk in broken out eggs of 2-3 weeks of age failed to increase in numbers during 5 days of incubation at ambient temperature. In most instances those placed next to the yolk membrane remained in the lag phase for 2 days before increasing from 10^3-10^9 organisms per egg during the 2-4 day period of incubation. They deduced that storage changed the properties of the yolk membrane (vitelline membrane ?) such that "nutrients or some factors which negated the inhibitory properties of the albumen" accumulated around the yolk and "reach(ed) a sufficiently high concentration to permit ... growth". In the apparatus illustrated in Figure 32 (Plates 1-3), it was demonstrated that the population size increased, - as indicated by clouding - only in the presence of the yolk. In its absence no clouding was observed. Likewise with *Ps.putida* the initial clouding was associated with albumen adjacent to the yolk. Many studies have shown that some -eg. elasticity-, but not all of the physical attributes -eg. the semipermeable property- of the

vitelline membrane change with time (Fromm 1967). In the egg at oviposition this membrane separates the yolk (freezing point -0.57 ^{0}C) from albumen (freezing point -0.42 ^{0}C), a function that is usurped by the yolk sac membrane during early embryogenesis. As the difference in freezing points equates to a difference in osmotic pressure of about 1.8 atm, one might expect a rapid flow of water from the albumen to the yolk. In practice this is prevented by the complex physical structure of the yolk emulsion (Burley and Vadehra 1989). With fertile eggs incubated for 8 hours at 37.5 ^{0}C, the glucose content (>50mM) in the albumen immediately surrounding the yolk was greater than that elsewhere in the albumen or yolk (Garcia *et al.* 1983). The reverse situation obtained with amino acids (Pons *et al.* 1985). Their concentration throughout the yolk (>30mM Kg) was considerably greater than the peak concentration (<10mM Kg) which again occurred in the white adjacent to the yolk. Ducay *et al.* (1960) recorded differences of this magnitude of amino acids in infertile eggs on the day of lay. They noted also that there was a very small but progressive increase in the concentration of these acids (0.42 to 1.34mM amino acid CO_2/ml albumen) during the storage of eggs for 20 days at 25 ^{0}C. Pons *et al.* (1985) demonstrated that amino acids were bound to albumen proteins. Thus, their concentration in the albumen surrounding the yolk would be expected to increase disproportionately as a consequence of diffussion. This feature obviously needs further attention in view of the

contentions of Humphrey *et al.* (1991). Their assertion that "some factors ... negate the inhibitory properties of albumen" could be taken to mean that Fe^{3+} diffuses from the yolk. Many studies (eg. Tranter and Board 1982) have shown that iron is an essential supplement if large bacterial populations are to develop in albumen *in vitro* and that combined nitrogen, whether organic or inorganic, makes a paltry contribution. This was confirmed in the study of the influence of various supplements on *S.enteritidis* in albumen *in vitro.* The outward diffusion of iron has attracted little attention. Circumstantial evidence suggests that it is probably of negligible importance. Thus the greenish colour caused by the reaction of Fe^{3+} and sulphur compounds is confined to the surface of the yolk of old eggs that have been hard boiled (Tinkler and Soar 1920). Indeed Schaible *et al.* (1944) found that the iron concentration of albumen increased to a limited extent only (from 74 to 118 micrograms/100 g albumen) in eggs stored at chill temperatures for 7.5 months. Copious accumulation of Fe^{3+} in the albumen with the development of a pronounced amber colour does not occur unless the complex structure of the yolk noted above is damaged as happens when yolks are frozen at temperatures above -6 ^{0}C.

Rapid iron migration from the yolk is a feature of eggs laid by hens receiving diets containing improperly prepared cotton seed meal or mallow seeds (Shenstone 1968). In this case one or other of 2 fatty acids, malvalic and sterculic, containing a cyclic propene group

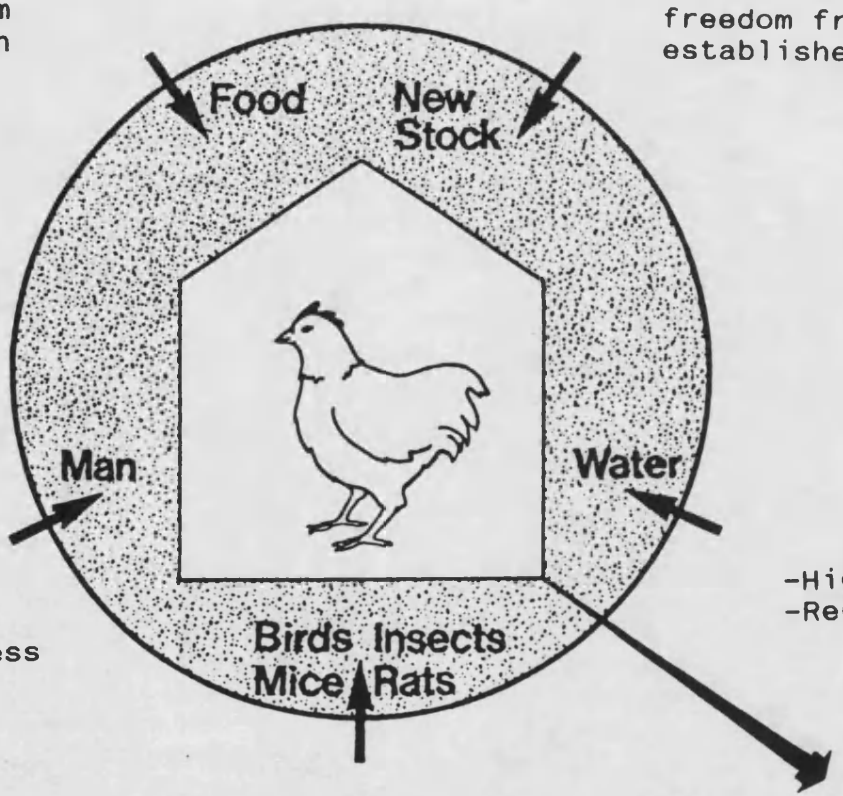
have been associated with changes in yolk structure and rapid iron migration. It needs to be stressed that migration is so rapid that "pink whites" develop and eggs with such faults are easily spotted. Consequently feed-induced changes in the rate of iron migration from the yolk is unlikely to have impinged upon the work of Humphrey *et al.* (1991) or that discussed in this report. In view of these observations it would seem reasonable to invoke a chemotactic response when discussing the results obtained by Humphrey *et al.* (1991).

When one considers the vast numbers of eggs produced (30 million eggs consumed/day in the UK - North and Gorman 1991) there would appear to be little hope of completely preventing egg transmission. It is therefore the obligation of egg marketing companies to control the spread of the organism in eggs by ensuring the rapid use of the product - especially in supermarkets where eggs may remain in the food chain for upto 5-6 weeks. Eggs must also be stored at chill temperatures, to prevent the growth of contaminating organisms. Lastly if cleaning methods are used, it must be ensured that they are carried out correctly. That is the incidence of egg infection must not be increased or growth of contaminating organisms induced - for example the washing of eggs in iron or faeces contaminated water.

Figure 39. Longitudinally Integrated Safety Assurance (LISA)

FEEDSTUFFS
-Pasteurization
-Supplementation with acid
-Protection from recontamination

-Bird selection for quality of albuminous sac and cuticle
-Antibiotic therapy
-Nurmi concept
-Isolation of stock until freedom from salmonellas established (100% cloacal swabs)



-Education
-Limited access

-High quality water
-Regular testing

-Good building material and design
-Well produced equipment
-Appropriate stocking density
-Isolation proceedures to keep out vermin
-Regular testing of the environment

Egg
-(1) Fumigation/Hatchery
-(2) or Washing ?
- Rapid movement from producer to consumer

References

Aasa. R, Malmstrom. B G, Saltman. P, Vanngard. T (1963) The specific binding of iron III and copper II to transferrin and conalbumin. Biochim Biophysica acta 75; 203-222.

Ager. E A, Enrad. E N, Galton. M M, Boring. J R, Jernigan. J R (1967) Two outbreaks of egg-borne salmonellosis and implications for their prevention. J Am Med Assoc 199; 372-378.

Aisen. P, Leibman. A, Reich. H A (1966) Studies on the binding of iron to transferrin and conalbumin. J Biol Chem 241; 1666-1671

Aisen. P, Leibman. A, Zweier (1978) Stachiometric and site characteristics of the binding of iron to human transferrin. J Biol Chem 253; 1930-1937.

Aisen. P, Koenig. S H, Schillinger. W E, Scheinberg. I H, Mann. K G, Fisk. W (1970) Absence of dimers and nature of iron binding in transferrin solutions. Nature (London) 226; 859-861.

Alder. J (1969) Chemoreceptors in bacteria. Science 166; 1588-1597.

Alder. J (1973) A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. J Gen Microbiol 74; 77-91.

Alderton. G, Ward. W H, Fevold. H L (1945) Isolation of lysozyme from egg white. J Biol Chem 157; 43-58.

Allen. T E, Grigg. G W (1957) Sperm transport in the fowl. Aust J Agric Res 8; 788-799.

Amy. P S, Morita. R Y (1983) Starvation-survival patterns of 16 freshly isolated open-ocean bacteria. Appl Environ Microbiol 45; 1109-1115.

Amy. P S, Pauling. C, Morita. R Y (1983) Recovery from nutrient starvation by a marine *Vibrio* spp. Appl Environ Microbiol 45; 1688-1690.

Amy. P S, Pauling. C, Morita. R Y (1983) Starvation-survival process of a marine vibrio. Appl Environ Microbiol 45; 1041-1048.

Anderson. C W, McSweeney. C J, Morgan. W P, Sugden. J H (1935) A series of outbreaks of food poisoning in Cardiff during a period of 4 months. Lancet 1; 285.

Anderson. E S, Threlfall. E J (1970) Change in a host range resistance factor. Genetic Res 16; 207-216.

Anon (1960) Widal method. In Mackie and McCartneys handbook of bacteriology. Ed Cruikshank. R. E & S livingstone Ltd (Edinburgh) p313.

Anon (1973) Salmonella infection in poultry. Advisory leaflet 298 (Scotland).

Anon (1975) Phenanthroline Method. In Standard Methods for the examination of water and wastewater. Ed Rand. MC, Greenberg. A E, Taras. M J. APHA-AWWA-WPCF. Washington p208-213.

Anon (1978) Egg quality - some recent research investigations. The eggs authority, technical bulletin number 2.

Anon (1981) The protein processing order.HMSO/MAFF (London).

Anon (1987) Update: *Salmonella enteritidis* infections in

the north-eastern United States. MMWR 36; 204-205.
Anon (1987) Increasing rate of *Salmonella enteritidis* infections in the north-eastern United States. MMWR 36; 10-11.
Anon (1987) Control measures for *Salmonella enteritidis* infections present and future efforts. CDC.
Anon (1987) Salmonella outbreaks. J Food Prot 51; 755.
Anon (1988) Salmonellosis associated with raw shell-egg. PHLS, CDR 88/21.
Anon (1989a) Memorandum of evidence to the Agriculture Committee inquiry on salmonella in eggs. PHLS Microbiology Digest 6; 1-9.
Anon (1989b) Zoonoses Order. HMSO/MAFF (London).
Anon (1991a) National Poultry Improvement Plan and Auxillary Provisions. United State department of Agriculture. Veterinary services - APHIS 91-40.
Anon (1991b) Sigma catalogue, Tissue culture p1530.
Anon (1992a) Ross Pedigrees Fly to new heights. Int Hatch Pract 6; 5-11.
Anon (1992b) Untouched by human hands ? Int Hatch Pract 6; 5-9.
Armstrong. R W, Fodor. T, Curlin. G T, Cohen. A B, Morris. G K, Martin, W T, Feldman. J (1970) Epidemic salmonella gastroenteritis due to contaminated ice cream. Am J Epidem 91; 300-307.
Asmundson. V S (1931) The formation of the hens egg. Scientific Agric 11; 672-680.
Ayres. J C, Taylor. B (1956) Effect of temperature on microbial proliferation in shell eggs. Appl Microbiol 4; 355-359.
Azari. P, Feeney. R E (1961) The resistances of conalbumin and its iron-complex to physical and chemical treatments. Arch Biochem Biophys 92; 44-53.
Baker. R G, Goff. J P, Mulnix. E-J (1980) Salmonellae recovery following oral and intravenous inoculation of laying hens. Poult Sci 59; 1067-1072.
Baker. R C, Goff. J P, Timoney. J F (1980) The prevalence of salmonellae on eggs from poultry farms in New York state. Poult Sci 59; 289-292.
Balls. A K, Swenson. T L (1934) The antitrypsin of egg white. J Biol Chem 106; 409-419.
Banwart. G J, Ayres. J C (1957) The effect of pH on the growth of salmonella and functional properties of liquid egg white. Food Tech 4; 244-246.
Barret. A J (1981) Cystatin, the egg white inhibitor of cysteine proteinases. Methods in Enzymol 80; 771-778.
Barnhart. H M, Dreesen. D W, Bastien. R, Pancorbo. O C (1991) Prevalence of *Salmonella enteritidis* and other serovars in ovaries of layer hens at time of slaughter. J Food Prot 54; 488-491.
Barrow. P A (1991) Immunological control of salmonella in poultry. In Colonization control of human bacterial enteropathogens in poultry. Ed Blankenship. L C. Academic Press Inc (London) p199-219.
Barrow. P A (1991) Experimental infection of chickens with *Salmonella enteritidis*. Avian Pathology 20; 145-153.
Baxter-Jones. C (1991) Egg hygiene: Microbial contamination,

significance and control. In Avian incubation. Ed Tullett. S G. Butterworth-Heinemann (London) p269-278.
Benjamin. W H, Briles. W E, Waltman. W D, Briles. D E (1991) Effect of genetics and prior *Salmonella enteritidis* infection on the ability of chickens to be infected with *Salmonella enteritidis*. In Colonization control of human bacterial enteropathogens in poultry. Ed Blankenship. L C. Academic Press Inc (London) p365-369.
Benson. C E, Eckroade. R J (1991) Virulence properties of *Salmonella enteritidis* isolates. In Colonization control of human bacterial enteropathogens in poultry. Ed Blankenship. L C. Academic Press Inc (London) p149-161.
Benson. C E, Eckroade. R J (1988) *Salmonella enteritidis* isolated from poultry related sources: Recommendations for isolation and characterization. JAUMA 192; 1784-1789.
Berg. H C, Brown. D A (1972) Chemotaxis in *Escherichia coli* analysed by three-dimensional tracking. Nature (London) 239; 500-504.
Berg. H C, Tedesco. P M (1975) Transient response to chemotactic stimuli in *Escherichia coli*. Proc Natl Acad Sci (USA) 72; 3235-3237.
Bigland. C H, Papas. G (1953) Experiment in egg penetration by salmonella. Canadian J Comp Med Vet Sci 17; 105-109.
Board. R G (1964) The growth of Gram-negative bacteria in the hens egg. J Appl Bact 27; 350-364.
Board. R G (1965) Bacterial growth on and penetration of the shell membranes of the hens egg. J Appl Bact 28; 197-205.
Board. R G (1966) Review article: The course of microbial infection of the hen's egg. J Appl Bact 29; 319-341.
Board. R G (1969) Microbiology of the egg: A review. In Egg quality. A study of the hens egg. Ed Carter. T C. Oliver & Boyd (Edinburgh) p133-162.
Board. R G, Ayres. J C (1965) Influence of temperature on bacterial penetration of hens eggs. Appl Microbiol 13; 358-364.
Board. P A, Board. R G (1967) A method of studying bacterial penetration of the shell of the hens egg. Lab Pract 16; 472-482.
Board. R G, Fuller. R (1974) Non-specific antimicrobial defences of the avian egg, embryo and neonate. Biol Rev 49; 15-49.
Board. R G, Halls. N A (1973) The effect of iron on the growth of *Escherichia coli* in albumen taken from the hens egg. Brit Poult Sci 14; 359-371.
Board. R G, Halls. N A (1973) The cuticle: A barrier to liquid and particle penetration of the shell of the hens egg. Brit Poult Sci 14; 69-97.
Board. P A, Hendon. L P, Board. R G (1968) The influence of iron on the course of bacterial infection of the hens egg. Brit Poult Sci 9; 211-215.
Board. R G, Sparks. N H C (1991) Shell structure and formation in avian eggs. In Egg incubation -its effect on embryonic development in birds and reptiles. Ed Deeming. D C, Ferguson. M W J. Cambridge University Press

(Cambridge) p71-87.
Boas. M A (1924) An observation on the value of egg white as the sole source of nitrogen for young growing rats. Biochem J 18; 422.
Bochner. B R, Savageau. M A (1977) Generalized indicator plate for genetic, metabolic and taxonomic studies with micro-organisms. Appl Environ 33; 434-444.
Bolder. N M, van Lith. L A J T, Mulder. R W A W (1991) Production of *Salmonella enteritidis* contaminated eggs after artificial inoculation of laying hens. In Proceedings 4th European symposium on the quality of eggs and egg products. Ed Oosterwoud. A, de Vries. A W p27-33.
Bowmer. E J (1965) Salmonellae in food - a review. J Milk Food Technol 28; 74-86.
Bradfield. J R G (1957) Radiographic studies on the formation of the hens egg shell. J Physiology 28; 125-140.
Brooks. J (1960) Mechanism of the multiplication of pseudomonas in the hen's egg. J Appl Bact 23; 499-509.
Brooks. J, Hale. H P (1959) The mechanical properties of the thick white of the hen's egg. Biochim Biophsyica acta 32; 237-250.
Brooks. J, Taylor. D I (1955) Eggs and egg products. Report of the Food Investigation Board 60. London HMSO.
Broomhead. C L, Mann. P G (1959) A family outbreak of *Salmonella typhimurium* infection. Mon Bull Ministry Health 18; 124-127.
Brown. D D, Ross. J G, Smith. A F G (1976) Experimental infection of poultry with *Salmonella infantis*. Res Vet Sci 20; 237-243.
Bruce. J, Drysdale. E M (1991) Egg hygiene: Routes of infection. In Avian Incubation, Poultry symposium 22. Ed Tullet. S G. Butterworth-Heinemann (London) p257-269.
Brus. I, Glass. G B J (1973) Estimation of cytotoxic injury to gastric parietal cells by Trypan blue exclusion test followed by Hematoxylin-Eosin counterstaining of fixed smears. Staining Tech 45; 127-132.
Buchanan. R E (1918) Life phases in bacterial culture. J Infect Dis 23; 109-125.
Büchli. K (1967) De inloed van het wassen op de microbiologische houdbaarheid van het ei. Veeleelt-en Zulvelberichten 10. 9/10.
Buck. J D (1977) The plate count in aquatic microbiology. In Native aquatic bacteria: Enumeration, activity and materials. Ed Costerton. J W, Colwell. R R. ASTM Special technical publications (Philadelphia).
Bullen. J J, Rogers. H J, Griffiths. E (1978) Role of iron in bacterial infection. Current topics in microbial immunolgy 80; 1-35.
Burley. R W, Vadehra. D V (1989) The avian egg: Chemistry and Biology. John Wiley & Sons (New York).
Butkevich. V S (1932) Zur methodik der bakteriolgischen Meeresunter-suchungen und einige Angaben uber die Verteilung der Bakterien im Wasser und in den Boden

des Barents Meeres. Trans Oceanogr Inst Moscow 2; 5-39.
Butkevich. N V, Butkevich. V S (1936) Multiplication of sea bacteria depending on the composition of the medium and on temperature. Microbiology (Moscow) 5; 322-343.
Buxton. A (1952) On the transference of bacterial antibodies from the hen to the chick. J Gen Microbiol 7; 268-286.
Byrd. J J, Xu. H, Colwell. R R (1991) Viable but non-culturable bacteria in drinking water. Appl Environ Microbiol 57; 875-878.
Chai. T J (1983) Characteristics of *Escherichia coli* grown in bay water as compared with rich medium. Appl Environ Microbiol 45; 1316-1323.
Chapman. P A, Rhodes. P, Rylands. W (1988) *Salmonella typhimurium* phage type 141 infections in Sheffield during 1984-1985: Association with hens' eggs. Epidem Infect 101; 75-82.
Chart. H, Rowe. B, Baskerville. A, Humphrey. T J (1990) Serological response of chickens to *Salmonella enteritidis*. Epidem Infect 104; 63-71.
Chung. K C, Goepfer. J M (1970) Growth of salmonella at low pH. J Food Sci 35; 326-328.
Clark. C W, Ordal. Z J (1969) Thermal injury and recovery of *Salmonella typhimurium* and its effect on enumeration procedures. Appl Microbiol 18; 332-336.
Clay. C E, Board. R G (1991) Growth of *Salmonella enteritidis* in artificially contaminated hens' shell eggs. Epidem Infect 106; 271-281.
Clay. C E, Board. R G (1992) Effect of faecal extract on the growth of *Salmonella enteritidis* in artificially contaminated hens' eggs. Brit Poult Sci 33; 755-760.
Collins. F M, Carter, P B (1972) Comparative immonogenicity of heat-killed and living oral *Salmonella* vaccines. Infect Immun 6; 451-458.
Collins. F M, Milne. M (1966) Heat-labile antigens of *Salmonella enteritidis* II. Mouse-protection studies. J Bact 92; 549-557.
Colwell. R R (1991) Viable but non-culturable bacteria in the aquatic environment. Culture (Oxoid) 12; 2-5.
Cotterill. O J, Winter. A R (1954) Egg white lysozyme 3. The effect of pH on the lysozyme ovomucin interaction. Poult Sci 34; 679-686.
Coughlan. M P (1971) The role of iron in microbial metabolism. Sci Prog Oxford. 59; 1-23.
Cowden. J M, Chisholm. D, O'Mahoney. M, Lynch. D, Mawer. S L, Spain. G E, Ward. L, Rowe. B (1989) Two outbreaks of *Salmonella enteritidis* phage type 4 infection associated with the consumption of fresh shell-egg products. Epidem Infect 103; 47-52.
Cowden. J M, Lynch. D, Joseph. C A, O'Mahoney. M, Mawer. S L, Rowe. B, Bartlett. C L R (1989) Case control study of infections with *Salmonella enteritidis* phage type 4 in England. B M J 299; 771-773.
Cox. N A, Davis. B H, Watts. A B, Colmer. A R (1973) Salmonella in the laying hen 1. *Salmonella* recovery from viscera, faeces and eggs following oral

inoculation. Poult Sci 52; 661-666.
Cox. G B, Gibson. F, Luke. R K J, Newton. N A, O'Brien. I G, Rosenberg. H (1970) Mutations affecting iron transport in *Escherichia coli*. J Bact 104; 219-226.
Coyle. E F, Ribeiro. A J, Howard. A J, Palmer. S R, Jones H I, Ward. L, Rowe. B (1988) *Salmonella enteritidis* phage type 4 infection: Association with hen's eggs. Lancet 8623; 1295-1296.
Cutler. R G, Evans. J E (1966) Synchronization of bacteria by the stationary phase method. J Bact 91; 469-476.
Dadrast. H, Hesketh. R, Taylor. D J (1990) Egg yolk antibody detection in identification of *Salmonella* infected poultry. Vet Rec 126; 219.
D'Aoust. J Y, Stotland. P, Randall. C J (1980) *Salmonella* in "Grade Cracks" shell eggs. Can Inst Food Sci Tech J 13; 184-187.
Dawes. E A (1976) Endogenous metabolism and the survival of starved prokaryotes. In Survival of vegetative microbes, 26th symposium of Soc Appl Bact. Ed Gray. T R G, Postgate. J R. Academic Press (Cambridge).
Dawes. E A (1984) Stress of unbalanced growth and starvation in micro-organisms. In The revival of injured microbes. The Soc Appl Bact Symp 12. Ed Andrew. M H E, Russell. A D. Academic Press (London) p19-20.
Dawes. E A (1985) Starvation survival and energy reserves. In Bacteria in their natural environments. Symposium of S G M 99th Ordinary meeting. Ed Fletcher. M, Floodgate. G. Academic Press (London) p43-79.
Derenzis. F A, Schechtman. A (1973) Staining by neutral red and Trypan blue in sequence for assaying vital and non-vital cultured cells. Stain Tech 48; 135-136.
Dolman. J, Board. R G (1992) The influence of temperature on the behaviour of mixed bacterial contamination of the shell membrane of the hen's egg. Epidem Infect 108; 115-121.
Druilhet. R E, Sobek. J M (1976) Starvation survival of *Salmonella enteritidis*. J Bact 125; 119-124.
Drysdale E M (1985) Microbial penetration of the avian egg. Msc Thesis. University of Glasgow.
Ducay. E D, Kline. L, Mandeles. S (1960) Free amino acid content of infertile chicken eggs. Poult Sci 39; 831-835.
Duguid. J P, North. R A E (1991) Eggs and salmonella food poisoning; an evaluation. J Med Microbiol 34; 65-72.
Dutton. R J, Bitton. G, Koopman. B (1983) Malachite green-INT (MINT) method for determining active bacteria in sewage. Appl Environ Microbiol 46; 1263-1267.
Dzierjowski. S K (1901) Contribution à l'étude de l'hérédité dans l'immunité artifielle *vis-a-vis* de la diphthérie. Arch Sci Biol (St Petersburg) 8; 429-440.
Eakin. R E, Snell. E E, Williams. R J (1940) The concentration and assay of avidin. The injury-producing protein in the raw egg white. J Biol Chem 140; 535-543.
Eichholz. A (1898) The hydrolysis of proteids. J Physiol 23; 163-177.

Elliot. R P (1954) Spoilage of shell eggs by pseudomonads. Appl Microbiol 2; 158-163.
Englemann. T W (1883) Bacterium photometricum. En beitrag zur vergleichenden physiologie des licht-ind. farbensinnes. Pflugers Archive fur gesammte Physiologie 95-124.
Fleming. A (1922) On a remarkable bacteriolytic element found in tissues and secretions. Pro Royal Soc London 93B; 306-317.
Forsythe. R H, Ayres. J C, Radlo. J L (1953) Factors affecting the microbiological populations of shell eggs. Food Tech 7; 49-66.
Forsythe. R H, Ross. W J, Ayres. J C (1967) *Salmonellae* recovery following gastro-intestinal and ovarian inoculation in the domestic fowl. Poult Sci 46; 849-855.
Fossum. K, Whittaker. J R (1968) Ficin and papain inhibitor from chicken egg white. Archives of Biochem Biophys 125; 367-375.
Francisco. D E, Mah. R A, Rabin. A C (1973) Acridine orange-epifluorescence technique for counting bacteria in natural waters. Trans Amer Micros Soc 92; 416-421.
Fromm. D (1967) Some physical and chemical changes in the vitelline membrane of the hen's egg during storage. J Dairy Sci 32; 52-56.
Fromm. D, Monroe. R J (1960) Inferior quality and bacterial contamination of market eggs as influenced by egg shell permeability. Food Tech Champaign 14; 401.
Frost. J A, Ward. L R, Rowe. B (1989) Acquisition of a drug resistance plasmid converts *Salmonella enteritidis* phage type 4 to phage type 24. Epied Infect 103; 243-248.
Garcia. F J, Pons. A, Alemany. M, Palou. A (1983) Permeability of chicken egg vitelline membrane to glucose, carbohydrate gradients between albumen and yolk. Comp Biochem Physiol 75B; 137-140.
Garibaldi. J A (1960) Factors in egg white which control growth of bacteria. Food Res 25; 337-344.
Garibaldi. J A (1970) Role of microbial iron transport compounds in the bacterial spoilage of eggs. Appl Microbiol 20; 558-560.
Garibaldi. J A (1972) Influence of temperature on the biosynthesis of iron transport compounds by *Salmonella typhimurium.* J Bact 110; 262-265.
Garibaldi. J A, Bayne. H G (1960) The effect of iron on the pseudomonas spoilage of experimentally infected shell eggs. Poult Sci 39; 1517-1520.
Garibaldi. J A, Stokes. J L (1958) Protective role of shell membranes in bacterial spoilage of eggs. Food Res 23; 283-290.
Gast. R K, Beard. C W (1990) Production of *Salmonella enteritidis* contaminated eggs by experimentally infected hens. Avian Dis 34; 438-446.
Gast. R K, Beard. C W (1990) Current ARS research on *Salmonella enteritidis* in chickens; Experimental infections in laying hens. Dairy Food Environ Sanitation 10; 267-278.

Gast. R K, Beard. C W (1992) Detection and enumeration of *Salmonella enteritidis* in fresh and stored eggs laid by experimentally infected hens. J Food Prot 55; 152-156.
Germanier. R (1972) Immunity in experimental salmonellosis III. Comparative immunization with viable and heat-inactivated cells of *Salmonella typhimurium.* Infect Immun 5; 792-797.
Gillespie. J M, Scott. W J (1950) Studies of shell eggs IV. Experiments on the mode of infection by bacteria. Aust J Appl Sci 1; 514-530.
Gottschal. J C (1992) Substrate capturing and growth in various ecosystems. J Appl Bact Sym Sup 21; 39-49.
Greenblatt. A P, Delay. P, Breslow. L, Greenblatt. J (1946) Salmonella epidemic from commercially prepared sandwiches. The Bulletin of the U S Army medical Dept. 5; 345-346.
Griffiths. E, Stevenson. P, Thorpe. R, Chart. H (1985) Naturally occurring antibodies in human sera that react with the iron-regualted outer membrane proteins of *Escherichia coli.* Infect Immun 47; 808-813.
Grimes. D J, Atwell. R W, Brayton. P R, Palmer. L M, Rollins. D M, Rozak. D B, Singleton. F L, Tamplin. M L Colwell. R R (1986) The fate of pathogenic bacteria in estuarine and marine environments. Microbiol Sci 3; 324-329.
Groat. R G, Schultz. E, Zychlinsky. E, Bockman. A, Matin. A (1986) Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. J Bact 168; 486-493.
Gunn. R A, Markakis. G (1978) Salmonellosis associated with homemade ice cream. J Am Med Assoc 240; 1885-1886.
Gurr. E (1965) The rational use of dyes in biology and general staining methods. Leonard Hill (London) p318-320.
Guzzewich. J J (1988) Emerging trends: *Salmonella enteritidis.* Assoc Food Drug Officials 50-54.
Haines. R B, (1939) Microbiology in the preservation of the hens egg. Dept of Scientific and Industrial Res. Food Investigation. Special Report 47. London HMSO.
Haines. R B, Moran. T (1940) Porosity of, and bacterial invasion through the shell of the hens egg. J Hygiene 40; 435-461.
Hammond. J (1940) Farm animals their breeding, growth and inheritance. London.
Harder. W, Dijkhuizen. L (1983) Physiological responses to nutrient limitation. Ann Rev Microbiol 37; 1-23.
Harry. E G (1963) Some observations on the bacterial content of the ovary and oviduct of the fowl. Brit Poult Sci 4; 63-70.
Harry. E G (1963) The relationship between egg spoilage and the environment of the egg when laid. Brit Poult Sci 4; 91-100.
Harvey. R W S, Price. T H (1961) An outbreak of salmonella food poisoning attributed to bakers confectionery. J Hyg Camb 59; 105-107.
Hassan. J O (1991) The immunological basis of chickens protection against *Salmonella typhimurium* F98. Colonization control of human enteropathogens in

poultry. Ed. Blankenship. L C. Academic Press Inc (London) p277-287.
Hazelbaur. G L, Parkinson. J S (1977) Bacterial chemotaxis. In Microbial interaction Series B, 3. Ed Reissig J C. John Wiley & Son Inc (New York) p60-98.
Hehewerth. F H (1901) Die mikroscopische zahlungsmethode der bacterien von Alex Klein und einige anwendungen derselben. Arch P Hyg 39; 321-330.
Hightower. L E (1980) Cultured animal cells exposed to amino acid analogues of puromycin rapidly synthesize several polypeptides. J Cell Phys 102; 407-427.
Hinton. M, Linton. A H, Perry. F G (1985) Control of salmonella by acid disinfection of chicks' food. Vet Rec 116; 502.
Hinton. M, Pearson. G R, Threlfall. E J, Rowe. B, Woodward. M, Wray. C (1989) Experimental *Salmonella enteritidis* infection in chicks. Vet Rec 124; 223.
Hinton. M, Threlfall. E J, Rowe. B (1990) The invasive potential of *Salmonella enteritidis* phage types for young chickens. Lett Appl Microbiol 10; 237-239.
Hirsch. P, Rapporteur. M, Bernhard. S S, Cohen. J C, Ensign. H, Jannasch. H W, Koch. A L, Marshall. K C, Martin. A, Poindexter. J S, Rittenberg. S C, Smith. D C, Veldkamp. H (1979) Life under conditions of low nutrient concentrations. Group report. In Strategies of microbial life in extreme environments. Ed Shilo. M. Dahlem Konferenzen (Berlin) p357-372.
Hobbie. J E, Daley. R J, Jasper. S (1977) Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33; 1225-1228.
Holl. A (1965) Vital stain by Trypan blue; its selectivity. Stain Tech 40; 269-273.
Hood. M A, Guckert. J B, White. D C, Deck. F (1986) Effect of nutrient deprivation on lipid, carbohydrate, DNA, RNA and protein levels in *Vibrio cholerae*. Appl Environ Microbiol 52; 788-793.
Hopper. S A, Mawer. S (1988) *Salmonella enteritidis* in a commercial layer flock. Vet Rec 123; 351.
Horowitz. J (1903) Jahresbreicht under die fort schritte der lehre von don pathogen. Mikkoorga 19; 984-985.
Horox. N (1989) Salmonella monitoring - What is meaningful ? Int Hatch Pract 3; 33-37.
Hoskin. J M, Meynell. G C, Sanders. F K (1956) A comparison of methods for estimating the viable count of a suspension of tumour cells. Exp Cell Res 11; 297-305.
Hughes. B O, Gilbert. A B (1984) Induction of eggshell abnormalities in domestic fowls by administration of adrenaline. IRCS Med Sci 12; 969-970.
Humphrey. T J (1991) *Salmonella enteritidis* PT 4 in laying hens and consequences for egg contamination. Proceedings 4th Symp on the quality of eggs and egg products. Ed Oosterwould. A, de Vries. A W. p9-17.
Humphrey. T J, Baskerville. A, Mawer. S, Rowe. B, Hopper. S (1989) *Salmonella enteritidis* phage type 4 from the contents of intact eggs: A study involving naturally infected hens. Epidem Infect 103; 415-423.

Humphrey. T J, Chart. H, Baskerville. A, Rowe. B (1991) The influence of age on the response of SPF hens to infection with *Salmonella enteritidis* PT 4. Epidem Infect 106; 33-43.
Humphrey. T J, Lanning. D G (1988) The vertical transmission of salmonellas and formic acid treatment of chicken feed. Epidem Infect 100; 43-49.
Humphrey. T J, Richardson. N P, Gawler. A H L, Allen M J (1991) Heat resistance of *Salmonella enteritidis* PT 4 the influence of prior exposure to alkaline conditions. Lett Appl Microbiol 12; 258-260.
Humphrey. T J, Whitehead. A, Gawler. A H L, Rowe. B (1991) Numbers of *Salmonella enteritidis* in the contents of naturally contaminated hens' eggs. Epidem Infect 106; 489-496.
Ichihara. S, Mizushima. S (1977) Involvement of outer membrane proteins in enterochelin mediated iron uptake in *Escherichia coli*. J Biochem 81; 749-756.
Jannasch. H W, Jones. G E (1959) Bacterial populations in sea water as determined by different methods of enumeration. Limnol Oceanogr 12; 128-39.
Jeffrey. F P, Pino. J (1943) The effects of heredity and of certain environmental factors on the incidence of blood spots in chicken eggs. Poult Sci 22; 230-234.
Jenkins. D E, Schultz. J E, Matin. A (1988) Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. J Bact 170; 3910-3914.
Johnston. F A (1956) Iron content of eggs. J Am Dietet Assoc 644-646.
Jones. F T, Axtell. R C, Rives. D V, Scheideler. S E, Tarver. F R, Walker. R L, Wineland. M J (1991) A survey of *Salmonella* contamination in modern brioler production. J Food Prot 54; 502-507.
Jones. F T, Axtell. R C, Tarver. F R, Rives. D V, Scheideler. S E, Wineland. M J (1991) Environmental factors contributing to *Salmonella* colonization of chickens. In Colonization control of human enteropathogens in poultry. Ed Blankenship. L C Academic Press (London) p3-23.
Kampelmacher. E M (1963) Public health and poultry products. Brit Vet J 119; 110-120.
Kato. A, Imoto. T, Yagishita. K (1975) The binding groups in ovomucin-lysozyme interaction. Agr Biol Chem 39; 541-544.
Kato. A, Sato. Y (1972) The release of carbohydrate rich component from ovomucin gel during storage. Agr Biol Chem 36; 831-836.
Kawakami. M, Osawa. N, Mitsuhashi. S (1966) Experimental salmonellosis VII. Comparison of the immunizing effect of live vaccine and materials extracted from *Salmonella enteritidis*. J Bact 92; 1585-1589.
Keung. W, Azari. P, Phillips. J L (1982) Structure and function of ovotransferrin. J Biol Chem 257; 1177-1183.
Khoury-Doughly. E, Spencer. J V, Verstrate. J A (1976) The effect of albumen on the iron requirements of *Salmonella oranienberg*. Poult Sci 55; 1124-1126.
Kim. C J, Emery. D A, Rinke. H, Nagaraja. K V, Halvorsen. D A (1989) Effect of time and temperature on growth of

Salmonella enteritidis in experimentally inoculated eggs. Avian Dis 33; 735-742.
Kjelleberg. S, Hermasson. M (1984) Starvation-induced effects on bacterial surface characteristics. Appl Environ Microbiol 48; 497-503.
Kjelleberg. S, Humphrey. B A, Marshall. K C (1983) Initial phases of starvation and activity of bacteria at surfaces. Appl Environ Microbiol 45; 978-984.
Klein. A (1900) Eine neue zahlungsmethode der bakterien. Centrlbl P Bakt 27; 834-835.
Knothe. H (1984) Epidemiological aspects of salmonella in man. J Food Sci Agic 35;639.
Knowles. N R (1971) Salmonellae in eggs and egg products. In British egg marketing board symposium 7. Poultry disease and world economy.
Koch. A L (1979) Microbial growth in low concentrations of nutrients. In Strategies in microbial life in extreme environments. Ed Shilo. M. Dahlem Konferenzen (Berlin) p261-279.
Kochan. I (1977) Role of siderophores in nutritional immunity and bacterial parasitism. In Micro-organisms and minerals. Ed Weinberg. Marcel Dekker Inc (New York).
Kogure. K, Simidu. U, Taga. N (1979) A tentative direct microscopic method for counting living marine bacteria. Can J Microbiol 25; 415-420.
Kramer. T T, Cho. H C (1970) Transfer of immunoglobulins and antibodies in the hen's egg. Immunology 19; 157-167.
Lake. P E (1966) Physiology and biochemistry of poultry semen. In Advances in reproductive physiology 1. Ed Mclaren. A. Logos Press (London) p93-123.
Lake. P E (1969) Factors affecting fertility. In The fertility and hatchability of the hens egg. Ed Carter. T C, Freeman. B M. Oliver & Boyd (Edinburgh) p67-79.
Lamson. G H (1909) Infection and preservation of eggs. Storrs agricultural experiment station. Storrs (Conn) Bulletin 55.
Laschtschenko. P (1909) Uber die keimtotende und entwicklungshemmende wirkung von huhnereiweib. Z Hyg Infektker 64; 419-427.
Leach. R M, Rucker. R B, Van Dyke. G P (1981) Egg shell membrane protein: a non-elastin desmosine isodesmosine - containing protein. Arch Biochem Biophys 207; 353-359.
Lebacq-Verheyden. A M, Vaerman. J P, Heremans. J F (1972) A possible homologue of mammalian IgA in chicken serum and secretions. Immunology 22; 165-175.
Lebacq-Verheyden. A M, Vaerman. J P, Heremans. J F (1974) Quantification and distribution of chicken immunoglobulins IgA, IgM and IgG in serum and secretions. Immunology 27; 683-692.
Lee. S S, Robinson. F M, Wang. H Y (1981) Rapid determination of yeast viability. Biotech Bioeng Symp 11; 641-649.
Leslie. G A, Clem. L W (1969) Immunoglobulins of the chicken. Phylogeny of Immunoglobulin structure

and function III. J Exp Med 130; 1337-1352.
Lewis. D L, Gattie. D K (1991) The ecology of quiescent microbes. Am Soc Microbiol 57; 27-32.
Lin. F Y C, Morris. G, Trump. D, Tilghman. D, Wood. P K Jackman. N, Israel. E, Libonatt. J P (1988) Investigation of an outbreak of *Salmonella enteritidis* gastroenteritis associated with consumption of eggs in restaurant chain in Maryland. Am J Epid 128; 839-844.
Lineweaver. H, Murray. C W (1947) Identification of the trypsin inhibitor of egg white with ovomucoid. J Biol Chem 171; 565-581.
Lister. S A (1988) *Salmonella enteritidis* in broilers and broiler breeders. Vet Rec 123; 350.
Lorenz. F W (1966) Behaviour of spermatozoa in the oviduct in relation to fertility. In Physiology of the domestic fowl. Ed Horton-Smith. C, Amorosos. E C. Oliver & Boyd (London) p39-43.
Lugtenberg. B, Peters. R, Bernheimer. H, Berendsen. W (1976) Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. Molec Gen Genet 147; 251-262.
Lutsky. I T, Bell. A E (1953) Antibacterial action of egg albumen in embryonic immunity. Poult Sci 32; 285-293.
Macnab. R M (1976) Motility and chemotaxis. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology 1. Ed Neidhart. C, Ingraham. J L, Brooks. K, Magasanik. B, Schaechter. M. American Soc for Microbiol (Washington D C) p732-751.
MacNab. R M (1978) Bacterial motility and chemotaxis: The molecular biology of a behavioural system. Critical Rev Biochem 3; 291-341.
Madden. J M (1990) *Salmonella enteritidis* contamination of whole chicken eggs. Dairy Food Environ Sanitation 10; 268-270.
Mandelstam. J (1958) Turnover of protein in growing and non-growing populations of *Escherichia coli*. Biochem J 69; 110-119.
Martin. P, Mcleod. R A (1984) Observations on the distinction between oligotrophic and eutrophic marine bacteria. Appl Environ Microbiol 47; 1017-1022.
Mason. D, Vines E (1989) Contaminated feed for sale. New Scientist 120; (1643) 29.
Matin. A, Auger. E A, Blum. P H, Schultz. J E (1989) Genetic basis of starvation survival in non-differentiating bacteria. Ann Rev Microbiol 43; 293-316.
Matsumura. P, Roman. S, Volz. K, Mcnally. D (1990) Signalling complexes in bacterial chemotaxis. In Biology of the chemotactic response. Ed Armitage. J P, Lackie. J M. Cambridge University Press (Cambridge) p135.
Matsushima. K (1953) An undescribed trypsin inhibitor in egg white. Science 127; 1178-1179.
Mawer. S L, Spain. G E, Rowe. B (1989) *Salmonella enteritidis* phage type 4 and hens eggs. The Lancet 8632; 280.
Mayes. F J, Takebaili. M A (1983) Microbial contamination of the hens egg: A review. J Food Prot 46; 1092-1098.
McLeod. S, Barrow. P A (1991) Lipopolysaccharide-specific IgG in egg yolk from 2 chicken flocks infected with

Salmonella enteritidis. Lett Appl Microbiol 13; 294-297.
McIntosh. M A, Earhart. C F (1977) Co-ordinate regulation by iron of the synthesis of phenolate compounds and three outer membrane proteins in *Escherichia coli*..J Bact 131; 331-339.
Mead. G C, Impey. C S (1984) Application of the competitive exclusion principle for preventing salmonella colonization in the newly hatched chick. J Sci Food Agric 35; 632-639.
Meada. K, Imae. Y, Shioi. J, Oosawa. F (1976) Effect of temperature on motility and chemotaxis of *Escherichia coli*. J Bact 127; 1039-1046.
Melton. T, Hartman. P E, Stratis. J P, Lee. T L, Davis. A T (1978) Chemotaxis of *Salmonella typhimurium* to amino acids and some sugars. J Bact 133; 708-716.
Mesibov. R, Ordal. G W, Alder. J (1973) The range of attractant concentrations for bacterial chemotaxis and the threshold and size of response over this range. J Gen Physiol 62; 203-205.
Miles. A A, Khimji. P L (1975) Enterobacterial chelators of iron: Their occurrence, detection, and relation to pathogenicity. J Med Micro 8; 477-490.
Miller. W A, Crawford. L B (1953) Some factors influencing bacterial penetration of eggs. Poult Sci 32; 303-304.
Mitchell. E, O'Mahoney. M, Lynch. D, Ward. L R, Rowe. B, Uttley. A, Rogers. T, Cunningham. D G, Watson. R (1989) Large outbreak of food poisoning caused by *Salmonella typhimurium* definitive type 49 in mayonnaise. B M J 298; 99-101.
Mishu. B, Griffin. P M, Tauxe. R V, Cameron. D N, Hutcheson. R H, Schaffner. W (1991) *Salmonella enteritidis* gastroenteritis transmitted by intact chicken eggs. Annals of Internal Med 115; 190-194.
Mitsuhashi. S, Osawa. N, Saito. K, Kurashige. S (1966) Cellular immunity with special reference to monocytes of mice immunized with live vaccine of *Salmonella enteritidis*. Tohoku J Exp Med 89; 307-314.
Moats. W A (1978) Egg washing - A review. J Food Prot 41; 919-925.
Moats. W A (1979) The effect of washing eggs under commercial conditions on bacterial loads on egg shells. Poult Sci 58; 1228-1233.
Morita. R Y (1982) Starvation-survival of heterotrophs in the marine environment. In Advances in microbial ecology 6. Ed Marshall. K C. Plenum Publishing Corp (New York) p171-198.
Morita. R Y (1985) Starvation, and miniturization of heterotrophs with special emphasis on the maintenance of the starved viable state. In Bacteria in the natural environment: The effects of nutrient conditions. Ed Fletcher. M, Floodgate. G. Academic Press Inc (New York) p111-130.
Morita. R Y (1988) Bioavailability of energy and its relationship to growth and starvation survival in nature. Can J Microbiol 34; 436-441.
Morris. G K (1990) *Salmonella enteritidis* and eggs: Assessment of risk. Dairy Food Environ Sanitation 10; 279-281.

Morris. G K, McMurray. B L, Galton. M M, Wells. J G (1969) A study of the dissemination of salmonellosis in a commercial broiler chicken operation. Am J Vet Res 30; 1413-1421.

Mossel. D A A, Struijk. C B (1992) The contribution of microbial ecology to management and monitoring of the safety, quality and acceptability (SQA) of foods. Soc Appl Bact Symp 21. Blackwell Scientific (London) p1-23.

Mossel. D A A, Van Netten. P (1984) Harmful effects of selective media on stressed micro-organisms: Nature and remedies. In The revival of injured microbes. Ed Andrew. M H E, Russell. A D. The Soc for Appl Bact Symp 12. Academic Press (London) p329-369.

Munniyappa. K, Adiga. P R (1979) Isolation and characterisation of thiamin-binding protein from chicken egg white. Biochem J 177; 887-894.

Nagaraja. K V, kim. C J, Kumar. M C, Pomeroy. B S. (1991) Is vaccination a feasible approach for the control of salmonella ? In Colonization control of human bacterial enteropathogens in poultry. Ed Blankenship. L C. Academic Press (London) p243-254.

Nascimento. V P (1991) The ease of translocation of *Salmonella enteritidis* through the eggshell wall: an ultrastructural study. Proceedings 4th European symposium on the quality of eggs and egg products. Ed Osterwoud. A, de Vries. A W p19-26.

Neilands. J B (1974) Microbial iron metabolism - a comprehensive treatise. Ed Neiland. J B. Academic Press Inc (London).

Neumeister. A (1890) J Am Chem Soc 22; 422-450. Quoted in Osborne and Campbell (1900).

Newell. K W, Hobbs. B C, Wallace. E J G (1955) Paratyphoid fever associated with Chinese frozen whole egg. B M J 2; 1296-1298.

Nicholas. R A J, Andrews. S J (1991) Detection of antibody to *Salmonella enteritidis* and *Salmonella typhimurium* in the yolk of hens' egg. Vet Rec 128; 98-100.

Noble. R E (1935) The relative productivity of certain culture media. J Am Water works Assoc 27; 1143-1160.

North. R A E, Gorman. T (1990) Chickengate. An independent investigation of *Salmonella* in eggs scare. The IEA Health and Welfare unit (Great Britain).

Novitsky. J A, Morita. R Y, (1976) Morphological characterization of small cells resulting fron nutrient starvation of pyschrophilic marine *Vibrios*. Appl Environ Microbiol 32; 617-622.

Nurmi. E, Rantala. M (1973) New aspects of salmonella infection in broiler production. Nature (London) 241; 210-211.

O'Brien. J D P (1990) Aspects of *Salmonella enteritidis* control in poultry. World Poult Sci J 46; 119-124.

O'Brien. I G, Gibson. F (1970) The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*. Biochim Biophysica acta 215; 393-402.

O'Brien. I G, Cox. G B, Gibson. F (1971) Enterochelin hydrolysis and iron metabolism in *Escherichia coli*. Biochim Biophysica acta 237; 537-549.

Olsen. M W, Neher. B H (1948) The site of fertilization in the domestic fowl. J Exp Zoo 109; 355-366.
Ordal. G W (1976) Control of tumbling in bacterial chemotaxis by divalent cation. J Bact 126; 706-711.
Orel. V (1959) The *Pseudomonas* spoilage of eggs laid by individual hens. Poult Sci 38; 8-12.
Osborne. T B, Campbell. G F (1900) The protein constituents of egg. J Am Chem Soc 22; 422-450.
Ostlund. K (1971) Bacteriology of washed and unwashed eggs I. Bacterial counts of egg contents after storage. Acta Vet Scand 12; 467-478.
Padron. M N (1990) *Salmonella typhimurium* penetration through the eggshell of hatching eggs. Avian Dis 34; 463-465.
Palmer. S, Coyle. E (1989) *Salmonella enteritidis*. Lancet 8629; 99-100.
Pappenheimer. A J (1917) Experimental studies upon lymphocytes: The reactions of lymphocytes under various experimental conditions. J Exp Med 25; 633.
Paton. A M, Ayres. J C (1964) Use of fluorescent brightner for tracing the passage of salmonella in eggs. Nature 204; 803-804.
Patterson. R, Younger. J S, Weige. W O, Dixon. F J (1962) Antibody production and transfer to egg yolk in chickens. J Immun 89; 272-278.
Patterson. R, Younger. J S, Weige. W O, Dixon. F J (1962) The metabolism of serum proteins in the hen and chick and secretion of serum proteins by the ovary of the hen. J Gen Physiol 45; 501-513.
Perales. I, Garcia. M I (1990) The influence of pH and temperature on the behaviour of *Salmonella enteritidis* phage type 4 in home-made mayonnaise. Lett Appl Microbiol 10; 19-22.
Perales. I, Audicanea. A (1989) The role of hens' eggs in outbreaks of salmonellosis in north Spain. Int J Food Microbiol 8; 175-180.
Perez. J, Tello. O, Mata. M, Fuente. J (1986) Foodborne infections and intoxications - outbreaks evolution in Spain 1976-1984. In Proceedings of the Second World Congress on Foodborne Infections and Intoxications. Berlin p104-109.
Perry. R D, Weinberg. E D (1973) Effect of iron deprivation and temperature upon bacterial survival. Microbios 8; 129-135.
Pfeffer. W (1884) Locomotorische richtungsbewegungen durch chemische reize. Untersuchungen aus dem botanischen institut in Tubingen 1; 363-482.
Pfeffer. W (1888) Uber chemtaktische bewegunen von bacterien flagellaten, und volvocineen. Unter suchungen aus dem botanischen institut in Tubingen 2; 582-661.
Pfeffer.W (1904) Pflanzenphysiologie 2. Wilhem Engelman 798-814.
Philbrook. F R, MacCready. R A, Roekel. H V, Anderson. E S, Smyser. C F, Sanen. F J, Groton. W M (1960) Salmonellosis spread by a dietary supplement of avian source. New Eng J Med 263; 713-718.
Phillips. H J (1973) Dye exclusion tests for cell viability. In Tissue culture - methods and applications. Ed Kruse. P

F, Patterson. M K. Academic Press (London) p406-411.
Pivnick. H, Nurmi. E (1982) The nurmi concept and its role in control of salmonellae in poultry. In Developments in food microbiology 1. Ed Davis R. Applied Science Publishers (London) p41-70.
Pohl. P, Lintermans. P, Marin. M, Couturier. M (1991) Epidemiological study of *Salmonella enteritidis* strains of animal origin in Belgium. Epidemiol Infect 106; 11-16.
Poindexter. J S (1979) Morphological adaptations to low nutrient concentrations. In Strategies of microbial life in extreme environments. Ed Shilo. M. Dahlem Konferezen (Berlin) p341-356.
Pollack. J R, Neilands. J B (1970) Enterobactin an iron transport compound from *Salmonella typhimurium.* Biochem Biophys Res Comm 38; 989-993.
Pons. A, Garcia. F J, Palou. A, Lemany. M (1985) Permeability of chicken egg vitelline membrane to amino acids - binding of amino acids to egg proteins. Comp Biochem Physiol 82a; 289-292.
Poppe. C, Irwin. R J, Messiers. S, Finley. G G, Oggel. J (1991) The prevelence of *Salmonella enteritidis* and other *Salmonella* spp. among Canadian registered commercial chicken broiler flocks. Epidem Infect 107; 201-211.
Postgate. J R (1976) Death in macrobes and microbes. In Survival of vegetative microbes. Symp Soc Gen Microbiol 26. Ed Gray. T R G, Postgate. J R. Cambridge University Press (Cambridge) p1-18.
Postgate. J R, Crumpton. J E, Hunter. J R (1961) The measurement of bacterial viability by slide culture. J Gen Microbiol 24; 15-24.
Postgate. J R, Hunter. J R (1963) Acceleration of bacterial death by growth substrates. Nature (London) 198; 273.
Powell. E O (1956) A rapid method for determining the proportion of viable bacteria in a culture. J Gen Microbiol 14; 153-159.
Pritchard. D G, Nivas. S C, York. M D, Pomeroy. B S (1978) Effect of Gal-E mutant of *Salmonella typhimurium* an experimental salmonellosis in chickens. Avian Dis 22; 562-575.
Rahn. H, Ar. A, Paganelli. C V (1979) How birds eggs breathe. Scientific America 22; 79-82.
Rampling. A, Upson. R, Ward. L, Anderson. J, Peters. E, Rowe. B (1989) *Salmonella enteritidis* phage type 4 infection of broiler chickens a hazard to public health. Lancet 8660; 436-438.
Reeve. C A, Bockman. A T, Matin. A (1984) Role of protein degradation in the survival of carbon-starved *Escherichia coli* and *Salmonella typhimurium.* J Bact 157; 758-763.
Rettger. L F (1914) Ovarian infection in the domestic fowl and transmission of the disease to offspring. J Exp Med 19; 552-561.
Rizk. S S, Ayres. J C, Kraft. A A (1966) Effect of holding conditions on the development of salmonellae in artificially inoculated hen's eggs. Poult Sci 45; 825-829.
Robinson. D S, Monsey. J B (1972) Changes in the composition of ovomucin during liquefaction of thick egg white: The

effect of ionic strength and magnesium salts. J Sci Food Agric 23; 893-904.
Robinson. D S, Monsey. J B (1975) The composition and proposed subunit structure of egg-white ß ovomucin. Biochem J 147; 55-62.
Rodrigue. D C, Tauxe. R V, Rowe. B (1990) International increase in *Salmonella enteritidis*. A new pandemic ? Epidem Infect 105; 21-27.
Rollins. D M, Colwell. R R (1986) Viable but non-culturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl Environ Microbiol 52; 531-538.
Romanoff. A L, Hutt. F B (1945) New data on the origin of double avian eggs. Anat Rec 91; 143-154.
Romanoff. A L, Romanoff. A J (1949) The avian egg. John Wiley & Sons Inc (New York)
Rose. M, Orlans. E, Buttress. N (1974) Immunoglobulin classes in the hen's egg: Their segregation in yolk and white. European J Immun 4; 521-523.
Roszak. D B, Colwell. R R (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51; 365-379.
Roszak. D B, Grimes. D J, Colwell. R R (1984) Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. Canadian J Microbiol 30; 334-338.
Rowe-Byers. B R, Arceneaux. J E L (1976) Microbial transport of iron. In Microbiology. Ed Schlessinger. D. American Soc for Microbiol. (Washington D C).
Rubenstein. A D, Feemster. R F, Smith. H (1944) Salmonellosis as a public health problem in wartime. Am J Public Health 34; 841-853.
Ryan. F J (1959) Bacterial mutation in a stationary phase and the question of cell turnover. J Gen Microbiol 21; 530-549.
St.louis. M E, Morse. D L, Potter. M E, Demelfi. T M, Guzewich. JJ, Tauxe. R V, Blake. P A (1988) The emergence of Grade A eggs as a major source of *Salmonella enteritidis* infections. J Am Med Assoc 259; 2103-2107.
Säinz. F, Gonzalez. M, Roca. P, Alemany. M (1983) Physical and chemical nature of eggs from six breeds of domestic fowl. Brit Poult Sci 24; 301-309.
Salvat. G, Protais. J, Lahellac. C, Colin. P (1991) Excretion rate of *Salmonella enteritidis* in laying hens following production of *Salmonella enteritidis* contaminated eggs responsible for foodborne disease. Procedings 4th European symposium on the quality of eggs and egg products. Ed Oosterwoud. A, de Vries. A W p35-41.
Samson. R R, Mirtle. C, McClelland. D B L (1979) Secretory IgA does not enhance the bacteriostatic effects of iron-binding or vitamin B_{12} binding protein in human colostrum. Immunology 38; 367-373.
Sawicki. W, Kieler. J, Briand. P (1967) Vital staining with neutral red and trypan blue of ^{3}H-thymidine-labelled cells prior to autoradiography. Stain Tech 42; 143-146.
Schade. A, Caroline. L (1944) Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*,

Staphylococcus aureus, Escherichia coli and *Saccharomyces cerevisiae*. Science 100; (2584) 14-15.
Schade. A L, Reinhart. R w, Levy. H (1949) Carbon dioxide and oxygen in complex formation with iron and siderophilin the iron binding component of human plasma. Arch Biochem 20; 170-172.
Schaible. P J, Davidson. J A, Bandemer. P A (1944) The iron content of whites and yolks of eggs from hens fed iron supplements. Poult Sci 23; 441-445.
Schatt. K A, Myers. T J (1989) Avian intestinal immunity. Proceedings Avian Enter Dis Symposium. AAAP/AVMA Annual meeting.
Schneitz. C (1992) New approach to salmonella control. Int Hatch Pract 6; 33.
Schultz. J E, Matin. A (1988) Regulation of carbon starvation genes in *Escherichia coli*. In Homeostatic mechanisms in micro-organisms. Ed Whittenbury. R, Gould. G W, Banks. J G, Board. R G. Bath University Press (Bath).
Scott. W M (1930) Food poisoning due to eggs. B M J 2; 56-58.
Sesma. B, Alvarez. M J, Aramenda. P, Goni. B, Pabio de. N, Goni, P (1988) Aisalmiento extraintestinal de *Salmonella* en gallinas: estudio epidemiologico de dos brotes de salmonellosis por consumo de huevo crudo. Microbiologica Sema 4; 209-212.
Sharp. J C M (1989) Foodborne infections. Soc Gen Microbiol Quarterly 16; 50-51.
Sharp. P F, Whitaker. R (1927) The relation of the hydrogen ion concentration of egg white to its germicidal action. J Bact 14; 17-46.
Shenstone. F S (1968) The gross composition, chemistry and physico-chemical basis of organization of the yolk and white. In Egg Quality. A study of the hens egg. Ed Carter. T C. Oliver & Boyd (Edinburgh) p26-58.
Shivaprasad. H L, Timoney. J F, Morales. S, Lucio. B, Baker. R C (1990) Pathogenesis of *Salmonella enteritidis* infection in laying chickens 1. Studies on egg transmission, clinical signs, faecal shedding and serological responses. Avian Dis 34; 548-557.
Smeltzer. T L, Orange. K, Peel. B, Runge. G (1979) Bacterial penetration in floor and nest box eggs from meat and layer birds. Aust Vet J 55; 592-593.
Snoeyenbos. G H, Smyser. C F, Van Roekel. H (1969) Salmonella infection of the ovary and peritoneum of chickens. Avian Dis 13; 668-670.
Sparks. N H C (1992) The effect of egg washing on cuticle quality. Report for Ross Breeders Ltd.
Sparks. N H C, Board. R G (1984) Cuticle, shell porosity and water uptake through hens' eggshells. Brit Poult Sci 25; 267-276.
Sparks. N H C, Board. R G (1985) Bacterial penetration of the recently oviposited shell of hen's eggs. Aust Vet J 62; 169-170.
Staley. J T (1968) Prosthecomicrobium and Ancalomicrobium: New prosthecate fresh water bacteria. J Bact 95; 1921-1942.
Stavrick. S, Gleeson. T M, Blanchfield. B, Pivnick. H (1987)

Role of adhering microflora in competitive exclusion of *Salmonella* from young chicks. J Food Prot 50; 928-932.
Steere. A C, Craven. P J, Hall. W J, Leostakis. N, Wells. J G, Farmer. J F, Gangarosa. E J (1975) Person to person spread of *Salmonella typhimurium* after a hospital common source outbreak. Lancet 7902; 319-322.
Stevens. A, Joseph. C, Bruce. J, Fenton. D, O'Mahoney. M, Cunningham. D, O'Connor. B, Rowe. B (1989) A large outbreak of *Salmonella enteritidis* phage type 4 associated with eggs from overseas. Epidem Infect 103; 425-433.
Stokes. J L, Osborne. W W (1956) Effect of the egg shell membrane on bacteria. Food Res 21; 264-269.
Stokes. J L, Osborne. W W, Bayne. H G (1956) Penetration and growth of *Salmonella* in shell eggs. Food Res 21; 510-518.
Stuart. L S, McNally. E S (1943) Bacteriological studies on the egg shell. The role the shell plays in preventing infection of the albumen and yolk. US Egg Poult Mag 49; 28-47.
Sussman. A S, Halvarson. H O (1966) Spores their dormancy and germination. Harper & Row (New York).
Tan. A T, Woodworth. R C (1969) Ultra-violet difference spectral studies of conalbumin complexes with transition metal ions. Biochemistry 8; 3711-3716.
Taylor. J (1969) Salmonella and salmonellosis. In Bacterial Food Poisoning. Ed Taylor. J. The Royal Soc of Health p25-46.
Taylor. T G (1966) The endocrine control of calcium metabolism in the fowl. In Physiology of the domestic fowl. Ed Horton-Smith. C, Amoroso. E C. Oliver & Boyd (London) p199-202.
Telzak. E E, Budnick. L D, Zweig Greenberg. M S, Blum. S, Shayegani. M, Benson. C E, Schultz. S (1990) A nosocomial outbreak of *Salmonella enteritidis* infection due to the consumption of raw eggs. New Eng J Med 323; 394-397.
Tengerdy. C, Azari. P, Tengerdy. R P (1966) Immunochemical reactions of conalbumin and its metal complexes. Nature 211; 203-204.
Thain. J A, Wilding. G P, Cullen. G A (1984) Serological response of turkey hens to vaccination with *Salmonella hadar* and its effect on their subsequently challenged embryos and poults. Res Vet Sci 36; 320-325.
Threlfall. E J, Frost. J A (1990) A review: The identification, typing and fingerprinting of *Salmonella*, laboratory aspects and epidemiological applications. J Appl Bact 68; 5-16.
Tinkler. C K, Soar. M C (1920) The formation of ferrous sulphide in eggs during cooking. Biochem J 14; 114-119.
Torrella. F, Morita. R Y (1981) Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. Appl Environ Microbiol 41; 518-527.
Tranter. H S, (1982) The antimicrobial defence system of avian egg albumen. University of Bath PhD thesis.
Tranter. H S, Board. R G (1982) The antimicrobial defence of avain eggs: Biological perspective and chemical basis. J Appl Biochem 4; 295-338.

Tranter. H S, Sparks. N H C, Board. R G (1983) A note on the structure and iron binding properties of eggshell membranes. Brit Poult Sci 24; 123-130.
Truscott. R B (1981) Oral salmonella antigens for the control of salmonella in chickens. Avian Dis 25; 810-820.
Trussell. P C, Triggs. R E, Greer. B A (1955) Bacterial spoilage of shell eggs III. Farm practices promoting spoilage. Food Tech 9; 134-137.
Tyler. C (1953) Studies on eggshells II. A method for marking and counting pores. J Sci Food Agric 41; 266-272.
Tyler. C, Geake. F H (1953) Studies on egg shells I. The determination of membrane, pore and matrix-protein. J Sci Food Agric 4; 261-266.
Vadehra. D V, Baker. R C, Naylor. H B (1970) Infection routes of bacteria into chicken eggs. J Food Sci 35; 61-62.
Vairo. M L R (1961) Methylene blue solutions for staining dead yeast cells. Stain Tech 36; 329-330.
Van Demark. N L (1953) Mammalian germ cells. Ed Wolstenholme. G E W, Cameron. M P, Freeman J S. Little Brown (Boston) p159.
Van Drimmelen. G C (1945) Intraperitoneal insemination of birds. J Soc Agric Vet Med Assoc 16; 1-6.
Van Drimmelen. G C (1946) "Spermnests" in the oviduct of the domestic hen. J Soc Agic Vet Med Assoc 17; 1-11.
Van Drimmelen. G C (1951) Artificial insemination of birds by the intraperitoneal route. Onderspoort J Vet Res sup 1. Ed Alexander. R A. Union of South Africa Dept Agric. The government printer (South Africa) p22-35, 150-181.
Veldkamp. H, Jannasch. H W (1972) Mixed culture studies. J Appl Chem Biotechnol 22; 105-123.
Wade. H E, Morgan. D M (1954) Differentiation of growing and non-growing bacteria by a staining technique. Nature 174; 920-921.
Walden. C C, Allen. I V F, Trussell. P C (1956) The role of the egg shell and shell membranes in restraining the entry of micro-organisms. Poult Sci 35; 1190-1196.
Walton. A, Whetham. E O (1933) The survival of the spermatozoa in the domestic fowl. J Exp Biol 10; 204-211.
Waring. W S, Werkman. C H (1942) Growth in an iron-free medium. Arch Biochem 1; 303-310.
Warner. R C, Weber. I (1953) The metal combining properties of conalbumin. J Am Chem Soc 75; 5094-5101.
Warren. D C, Scott. H M (1935) The time factor in egg formation. Poult Sci 14; 195-207.
Watson. K (1989) Microbial stress proteins. In Advances in microbial physiology. Ed Rose A H, Tempest. D W. Academic Press (London) 31; 183-216.
Watt. J (1945) An outbreak of *Salmonella enteritidis* in man from infected chicken eggs. US Public health report (Washington) 60; 835-839.
Weinberg. E D (1979) Roles of iron in fever and infectious disease. In Thermoregulatory mecahnisms and their therapeutic implications. Ed Cox. B, Lomax. P, Milton. A S, Schoubaum. E. Karger Basel (Berlin) p105-110.
Williams. J (1974) The formation of iron-binding fragments of hen ovotransferrin by limited proteolysis. Biochem J

141; 745-752.
Williams. J E (1981) Salmonellas in poultry feeds - a worldwide review I, II, III. World Poult Sci J 37; 97-105.
Williams. J E, Whittemore. A D (1967) A method for studying microbial penetration through the outer structures of the avian egg. Avain Dis 11; 467-490.
Williams Smith. H, Tucker. J F (1980) The virulence of *Salmonella* strains for chickens their excretion by infected chickens. J Hyg Camb 84; 479-488.
Williams. S C, Woodworth. R C (1973) The interaction of iron-conalbumin (anion) complexes with chick embryo red blood cells. J Biol Chem 248; 5848-5853.
Wilson. G S (1922) The proportion of viable bacteria in young cultures with special reference to the technique employed in counting. J Bact 7; 405-446.
Wilson. J E (1948) Avian salmonellosis. Vet Rec 60; 615-622.
Winterberg. H (1898) Zur methodik der bakterienzahlung. Zeitschrift F Hyg U Infektionshrhtn 29; 75-93.
Worsham. P L, Konisky. J (1984) Effect of growth temperature on the aquisition of iron by *Salmonella typhimurium* and *Escherichia coli*. J Bact 158; 163-168.
Xu. H S, Roberts. N, Singleton. F L, Attwell. R W, Grimes. D J, Colwell. R R (1982) Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine environment. Microbiol Ecol 8; 313-323.
Yancey. R J, Breeding. S A, Lankford. C E (1979) Enterochelin: virulence factor for *Salmonella typhimurium*. Infect Immun 24; 174-180.
Young. I G, Cox. G B, Gibson. F (1967) 2,3-Dihyroxybenzoate as a bacterial growth factor and its route of biosynthesis. Biochim Biophysica acta 141; 319-331.
Zagaesvsky. J S, Lutikova. P O (1944) Bacterial contamination in eggs. US Egg Poult Mag 50; 75-89.
Zaske. S K, Dockins. W S, McFeters. A (1980) Cell envelope damage in *Escherichia coli* caused by short-term stress in water. Appl Environ Microbiol 40; 386-390.
Zelikow. J (1906) Quantitative bestimmung der bakterialmasse durch die kolorimetrische methode. Centrlbl f Bakt Abt Originale 425; 476-480.

APPENDIX 1

Phenanthroline method for iron determination

1). Stock iron solution - ml/L

Concentrated H_2SO_4	20
H_2O	50
$Fe(NH_4)_2(SO_4).6H_2O$	1.404

Add $KMNO_4$ dropwise until a faint pink colour develops.

Dilute to 1 l.

2). Hydroxylamine Hydrochloride solution

10g in 100ml H_2O

3). Sodium Acetate solution

25g in 100ml H_2O

4). Phenthroline solution

100mg in 100ml H_2O

5). Ammonium acetate buffer solution

Ammonium acetate	50g
H_2O	30ml
Glacial Acetic acid	140ml

6). Phenanthroline Monohydrate solution

100mg in 100ml H_2O

2 drops of concentrated HCl

NB All H_2O used is de-ionised.

Hanks Balance Salts Solution (g/l)

NaCl	8.0
KCl	0.4
Na_2PO_4	0.09
KH_2PO_4	0.06
$MgSO_4.7H_2O$	0.1
$CaCl_2$	0.14
Glucose	1.0
$NaHCO_3$	0.35
Phenol red	0.1

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Persistence of contamination of hens' egg albumen *in vitro* with *Salmonella* serotypes

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SUMMARY

A study was made of the persistence of different *Salmonella* serotypes in hens' egg albumen *in vitro* at 4, 20 and 30 °C. The majority of serotypes remained viable but did not increase in numbers at 20 and 30 °C for 42 days. At 4 °C many of the serotypes died out.

The addition of ferric ammonium citrate on the 42nd day of incubation induced multiplication of organisms incubated at 20 and 30 °C, but not at 4 °C. The pH and glucose concentration of the albumen diminished only when heavy growth occurred.

Salmonella enteritidis remained viable on the air cell membrane *in vitro* for 17 days at 4, 20 and 30 °C. Thirty percent of the organisms also remained motile in albumen for 42 days at 25 °C and up to 5% of the cells remained motile for up to 20 days at 4 °C.

INTRODUCTION

Salmonella enteritidis, predominantly phage type 4 (PT4), has been associated with outbreaks of salmonellosis in which eggs and egg products were the vehicles of transmission [1]. There are two possible routes of salmonella contamination of the egg contents. In one, contamination occurs before oviposition [2, 3]. The incidence is about 0·6% with naturally contaminated eggs, the level of contamination is low and the albumen is more frequently contaminated than the yolk [4]. In the other, organisms on the shell are translocated along the pore canals and lodged on the underlying shell membranes. Translocation may occur during egg washing if the wash water is cooler than the egg [5–7]. The temperature differential causes a slight negative pressure because the reduction in volume of the contents is greater than that of the shell [5]. This results in a small amount of contaminated water being sucked into an egg [5, 8]. In this instance the cuticle enveloping the outer surface of the calcite shell is the main impediment to water and bacterial translocation along the pore canals [9]. The immature cuticle on eggs at or for a few minutes following oviposition appears to be an ineffective barrier [9, 10]. Indeed, this may well explain the higher incidence of contamination of the contents of eggs laid on the floor of poultry houses over those laid in nest boxes [11–13]. Such studies have shown that a range of organisms gains access to the contents of eggs infected in this manner.

Many factors influence the behaviour of organisms that contaminate the shell membranes post trans-shell infection [14]. A recent study [15] showed that the storage temperature had a profound selective action on the members of a consortium of bacteria (*Pseudomonas putida*, *Staphylococcus xylosis*, *Enterococcus faecalis*, *Escherichia coli* and *Salmonella enteritidis* PT4) seeded on the membranes. *Pseudomonas putida* became the dominant organism in the shell membranes and eventually in the albumen of eggs stored at 4, 15 and 20 °C. In contrast, *Salmonella enteritidis* dominated the infection of both the shell membrane and contents of eggs stored at 37 °C. Clay and Board [16] noted that a pure culture of *S. enteritidis* was confined to the shell membranes of eggs stored at 4 °C. With storage at ambient temperature, there was a progressive increase in the number of eggs containing $> 1{\cdot}0 \times 10^7$ salmonellas per ml of albumen. They surmised that the salmonellas retained in the membrane and the initial invaders of the albumen did not grow because of the antimicrobial properties of the latter [14, 17] and that the large populations arose from the growth of contaminants which made contact with the yolk. Recently Humphrey and colleagues [4] concluded that contaminants which gain access to the albumen surrounding the yolk of eggs stored for 2–3 weeks grow on nutrients that have diffused from the yolk across the ageing vitelline membrane. It is evident that further work needs to be done to resolve these conflicting interpretations. The present study, which is a prelude to such investigations, examines the hypothesis that following trans-shell or oviducal infection of infertile hens' eggs the initial invaders of the albumen would not proliferate sufficiently to cause the gross contamination noted by Clay and Board [16].

MATERIALS AND METHODS

Eggs

Eggs (size 4, approx. 58 g) less than 2 days old were purchased from a local producer/retailer and stored for less than 2 days before use. Eggs were assumed to be salmonella-free at purchase. Eggs from the same source were used in other studies in which endogenous salmonellas would have been detected. None was found to be contaminated.

Cultures

The sources of the cultures are given in Table 1. These were stored on Dorset egg agar (Oxoid Ltd) at 4 °C and subcultured every 3 months. For experimental purposes, an overnight culture in nutrient broth (Lab M, incubated at 37 °C) was spun down (2000 g for 10 min), washed in saline (Lab M) and finally resuspended in the same medium and diluted such that 0·1 ml contained *c.* 10^3 organisms.

Persistence experiments

Eggshells were wiped with ethanol (70% v/v), cracked and the contents collected. Albumen and yolk were harvested and bulked separately. Seventy ml of blended albumen were dispensed into sterile containers (250 ml, Sterilin) and 0·1 ml of a cell suspension added. Duplicate samples were stored at 4, 20 or 30 °C and sampled regularly. Ferric ammonium citrate (BDH) was added (final concentration 0·008 mg/ml Fe^{3+}) on the 42nd day.

Viable counts were obtained by spreading 0·1 ml of an appropriate dilution on duplicate plates of Xylose Lysine Decarboxylase agar (XLD; Lab M) with overnight incubation at 37 °C. Presumptive salmonella colonies on XLD were confirmed serologically.

Initial and final glucose concentrations were determined (Boehringer Mannheim). The pH of the albumen was tested with Whatman indicator paper (range 1–14).

The in vitro *study of* S. enteritidis *on the inner shell membrane*

The air cells of eggs were located by candling. The shell was swabbed with 70% ethanol, a small hole drilled in the shell at this site and 0·1 ml of a *c.* 10^3 cell suspension injected onto the air cell membrane. The hole was sealed with paraffin wax and the eggs left at room temperature for *c.* 3 h. All the liquid was absorbed into the egg contents within 20 min of being applied to the shell membrane. Eggshells were then wiped with ethanol, cracked, the albumen separated and poured into sterile containers (250 ml Sterilin). The inoculated inner membrane of the air cell was excised from the shell and placed in the albumen.

The albumen was incubated at 4, 20 or 30 °C. Duplicate samples were tested for the presence of *S. enteritidis*.

Viable counts of the albumen were obtained on XLD. The membrane was plated directly onto XLD.

A motility index of organisms in albumen was obtained by comparing the number of motile cells and the number of non-motile ones. A minimum of 200 cells was counted per sample using × 1000 phase contrast microscopy.

RESULTS

The persistence of viable cells in albumen *in vitro* at 4, 20 or 30 °C was studied with 13 *Salmonella* serotypes and 13 phage types of *S. enteritidis* (Table 1). With the exceptions of *S. enteritidis* PT4 and *S. hadar*, and both on one occasion only, there was a progressive diminution in the viable counts in albumen at 4 °C such that our method of analysis failed (< 10 CFU/ml) to isolate organisms on the 42nd day of incubation. Microscopical examinations, which were done on every sampling occasion in one experiment with inoculated albumen at 4 °C, showed that up to 5% of *S. enteritidis* remained motile for 9–20 days. The addition of ferric ammonium citrate to albumen on the 42nd day of incubation at 4 °C did not induce multiplication of any of the *Salmonella* serotypes or phage types.

There was a spectrum of responses among salmonellas stored in unsupplemented albumen incubated at 20 or 30 °C. Eight of the 13 phage types of *S. enteritidis* (Table 1) multiplied sluggishly (generation time of days) at one or both of these temperatures (Fig. 1). When sluggish growth occurred neither the glucose content nor the pH of the albumen changed. It is evident from Fig. 1a that there was a very fast rate of growth following the addition of ferric ammonium citrate to albumen. When suspended in unsupplemented albumen at 25 °C, 30% of *S. enteritidis* PT4 cells were motile on the 42nd day of incubation, even though no demonstrable growth had occurred. The other phage types of this serotype merely persisted or their numbers diminished to undetectable levels in albumen at 20 or 30 °C. There was no demonstrable change in the glucose content or pH of the

Table 1. *The persistence of* Salmonella *serotypes in albumen* in vitro *with incubation at 4, 20 or 30 °C*

Salmonella serotype	Source*	Persistence at °C			Response to Fe^{3+}		
		4	20	30	4	20	30
enteritidis	A2†	+	+	+	ng	G	G
PT 4	A	−	g	g	ng	G	G
PT 4 cured	A	−	+	g	ng	G	G
PT 4a	B	−	g	(g)	ng	G	G
PT 1	B	−	g	g	ng	G	G
PT 6	B	−	g	(g)	ng	G	G
PT 13a	C	−	g	(g)	ng	G	G
PT 21	B	−	g	(g)	ng	G	G
PT 30	B	−	(g)	g	ng	G	G
PT 14b	B	−	(g)	(g)	ng	G	G
PT 5	B	−	(g)	(g)	ng	G	G
PT 12	B	−	(g)	+	ng	G	G
PT 8	C	−	(g)	−	ng	G	G
PT 24	B	−	+	+	ng	G	G
PT 23	B	−	+	−	ng	G	G
hadar	D2	+	+	(g)	ng	G	G
		−	(g)	(g)	ng	G	G
worthington	D	−	g	(g)	ng	G	G
waycross	E	−	(g)	+	ng	G	G
ohio	D	−	(g)	+	ng	G	G
brandenberg	E	−	+	+	ng	G	G
dublin	E	−	+	−	ng	G	G
infantis	E	−	+	−	ng	G	G
typhimurium	A	−	+	+	ng	G	G
montevideo	B	−	+	+	ng	G	G
senftenberg	D	−	+	+	ng	G	G
gallinarum	F	−	+	−	ng	G	G
pullorum	F	−	−	−	ng	G	G

* Source: A, Exeter PHLS; B, CVL (Weybridge); C, ex-egg USA; D, British United Turkeys; E, Bath University; F, Bristol University.

† All experiments done in duplicate, 2 indicates that 2 or more trials were conducted.

−, did not persist; +, persisted; (g), slight growth, generation time 13–19 days; g, growth, generation time 2–12 days; G, generation time 6–12 h.

albumen. Of the other serotypes, only *hadar* (Fig. 1b), *worthington*, *ohio*, and *waycross* multiplied in albumen at 20 or 30 °C. Again, however, the growth rate was sluggish and the results inconsistent. Thus, for example *S. hadar* merely persisted in two trials and grew in another (Table 1). In the latter instance the generation time was very long (> 13 days) such that there was only a 1000-fold increase in the population size during 42 days incubation of albumen at 20 °C. It only achieved a 100-fold increase at 30 °C.

Seven of the serotypes (*brandenberg*, *dublin*, *infantis*, *typhimurium*, *montevideo*, *senftenberg* and *gallinarum*) persisted but did not grow in albumen at 30 and/or 20 °C. The number of viable cells of *S. pullorum* diminished to undetectable levels at both of these temperatures.

Every strain of salmonella (Table 1) used in this study formed large populations

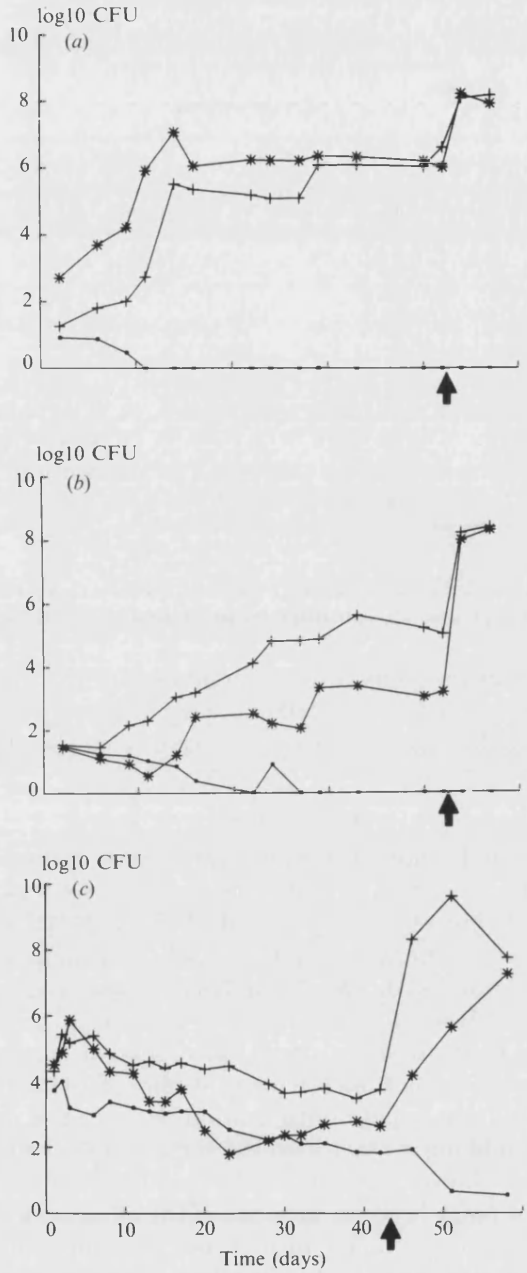

Fig. 1. The persistence of *S. enteritidis* (*a*) and (*c*), and *S. hadar* (*b*) at 4 °C, —●—; 20 °C, —+—; and 30 °C, —*—. Arrow indicates the addition of ferric ammonium citrate.

($> 1{\cdot}0 \times 10^7$) at 20 and 30 °C following the addition of ferric ammonium citrate to the albumen, e.g. Fig. 1c. It needs to be stressed that the generation times (< 12 h) in supplemented albumen was very short in contrast to those of *S. hadar*, for example, in unsupplemented albumen. The pH of the albumen changed (pH 9·5

Table 2. *The persistence of* Salmonella enteritidis *PT 4 in a piece of shell membrane in albumen* in vitro

	Persistence at			
	4 °C		25 °C	
Days	Membrane	Albumen	Membrane	Albumen
1	+	−	+	−
3	+	−	+	−
6	+	−	+	−
8	+	−	+	−
10	+	−	+	−
13	+	−	+	−
15	+	−	+	−
17	+	−	+	−

+, Organisms grew when membrane was placed on XLD and Nutrient Agar. −, No viable organisms grew from 0·1 ml of albumen placed on XLD and Nutrient Agar. The experiments were done in duplicate on two occasions.

to 7·0–8·0) when the populations of salmonellas attained $> 10^7$ cells/ml. Likewise the concentration of glucose in albumen diminished (50–70% loss) only when heavy growth occurred.

When *S. enteritidis* in the excised inner membrane of the air cell of eggs was suspended in albumen *in vitro*, it persisted in the membrane for upwards of 17 days at 4 and 25 °C. No viable organisms were recovered from the albumen (Table 2).

DISCUSSION

The studies by Clay and Board [16] demonstrated that, following inoculation of the inner shell membrane of the air cell with *S. enteritidis* PT4, there were two phases in the infection process of eggs stored at 10 or 25 °C. Persistence of the infection in the shell membrane together with contamination of albumen underlying the membrane with small numbers of quiescent organisms characterized the first phase. It was followed by rapid growth and gross infection of the albumen and yolk. Until recently there was general agreement based on circumstantial evidence coming from the many studies of infection of eggs with rot producing bacteria [18] that rapid multiplication occurs when organisms acquire essential nutrients on making contact with the surface of the yolk [19]. Humphrey and colleagues [4] have shown that salmonellas inoculated at the periphery of the albumen or at the mid point between the edge of the albumen and yolk in broken out eggs of 2–3 weeks of age failed to increase in numbers during 5 days of incubation at ambient temperature. In most instances those placed next to the yolk membrane remained in the lag phase for 2 days before increasing from 10^3 to 10^9 organisms per egg during the 2–4-day period of incubation. They deduced that storage changed the properties of the yolk membrane (vitelline membrane?) such that 'nutrients or some factors which negated the inhibitory properties of the albumen' accumulated around the yolk and 'reach(ed) a sufficiently high concentration to permit ... growth'. Future studies will need to address the changes occurring in the albumen of stored eggs and correlate these with the switch

from the quiescent to active growth phase of contaminating organisms. Further, such investigation could well aid interpretation of observations made in the present study.

We observed that non-proliferating salmonella inoculated into albumen remained motile for upwards of 42 days at 25 °C. The experiments with inoculated shell membranes suspended in albumen *in vitro* showed that the organism in the membranes remained viable but none was isolated from the albumen during 17 days incubation at 4 or 25 °C. These results, which are in accord with previous observations [20], lead us to suggest that organisms, particularly those in the infected shell membrane, move towards the food store of the yolk because of a gradient in the concentration of amino acids in the albumen. They multiply only when contact with the yolk provides other essential nutrients. Since the completion of this study we have obtained further evidence to support this hypothesis. These studies also suggested that the albumen's loss of viscosity, which occurs with storage, may facilitate microbial migration.

The present study has provided additional information on two subfeatures of the infection process immediately following trans-shell infection. Firstly, *Salmonella* serotypes and phage types exhibit a spectrum of response to egg albumen *in vitro*. It is noteworthy that among the organisms included in this study, *S. enteritidis* PT4 and *S. hadar*, both of which have been associated with infection of poultry and turkey flocks respectively [21], appear to be better adapted than the other serotypes in their capacity to remain viable or indeed grow feebly on some occasions in albumen at temperatures conducive to growth. Indeed, about a third of the *S. enteritidis* cells remained motile in unsupplemented albumen incubated at 25 °C for 42 days. The phenotypic attributes associated with this longevity and maintenance of motility are not known. It would be of interest to establish whether or not such attributes are also associated with those which cause *S. enteritidis* to be more invasive than other serotypes in young chicks [22].

Secondly, ferric ammonium citrate overcame the antimicrobial properties of the albumen for all 26 serotypes and phage types used in this study, providing incubation was at 20 or 30 °C. It has been shown repeatedly (see review by Tranter and Board [17]) that the bacteriostatic action of albumen against Gram-negative bacteria in general is negated by combined nitrogen and Fe^{3+} in amounts sufficient to saturate ovotransferrin. In such experiments the supplements were added before or immediately following inoculation of the albumen. In the present study bacteriostasis was released by supplements added on the 42nd day. It may be inferred, therefore, that the unsupplemented albumen induces a quiescent state in salmonella. This phenomenon was noted by Rozak, Grimes and Colwell [23] who worked on water contaminated naturally with this organism. This novel proposal may be linked with the observation [24] that the alkalinity (pH 9·6) of the albumen induces a marked change in the characteristics, e.g. increased heat resistance of these organisms.

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REFERENCES

1. Cowden JM, Lynch D, Joseph CA, et al. Case control study of *Salmonella enteritidis* phage type 4 in England. BMJ 1989; **299**: 771–3.
2. O'Brien, JDP. Aspects of *Salmonella enteritidis* control in poultry. World Poult Sci J 1990; ; **46**: 119–24.
3. Lister SA. *Salmonella enteritidis* infection in broilers and broiler breeders. Vet Rec 1988; ; **123**: 350.
4. Humphrey TJ, Whitehead A, Gawler AHL, Henley A, Rowe B. Numbers of *Salmonella enteritidis* in the contents of naturally contaminated hens' eggs. Epidemiol Infect 1991; ; **106**: 489–96.
5. Haines RB, Moran T. Porosity of and bacterial invasion through the shell of the hens' egg. *J Hyg* 1940; **40**: 453–61.
6. Büchli K. The effect of washing on the shelf life of eggs. Veeleelt-en Zuiveiberichten 1967; ; **10**: 9/10 (Sept/Oct).
7. Moats WA. Egg washing. A review. J Foot Prot 1978; **41**: 919–25.
8. Sparks NHC, Board RG. Cuticle, shell porosity and water uptake through hen's eggshells. Brit Poult Sci 1984; **25**: 267–76.
9. Sparks NHC, Board RG. Bacterial penetration of the recently oviposited shell of hens' eggs. Aust Vet J 1985; **62**: 169–70.
10. Vadehra DV, Baker RC, Naylor HB. Role of the cuticle in spoilage of chicken eggs. J Food Sci 1970; **35**: 5–6.
11. Smeltzer TI, Orange K, Peel B, Runge G. Bacterial penetration in floor and nest box eggs from meat and layer birds. Aust Vet J 1979; **55**: 592–3.
12. Padron MN. *Salmonella typhimurium* penetration through the shell of hatching eggs. Avian Dis 1990; **34**: 463–5.
13. Bruce J, Drysdale EM. Egg hygiene: routes of infection. In: Avian incubation. Poultry Science Symposium 22. Tullett SG, ed. London: Butterworth–Heinemann, 1991: 257–69.
14. Brooks J. Mechanism of the multiplication of *Pseudomonas* in the hens' eggs. J Appl Bacteriol 1960; **23**: 499–509.
15. Dolman J, Board RG. The influence of temperature on the behaviour of mixed bacterial infection of the shell membrane of the hens' egg. Epidemiol Infect 1991; **108**: 115–21.
16. Clay CE, Board RG. Growth of *Salmonella enteritidis* in artificially contaminated hens' shell eggs. Epidemiol Infect 1991; **106**: 271–81.
17. Tranter HS, Board RG. The antimicrobial defence of avian eggs: biological perspective and chemical basis. J Appl Biochem 1982; **4**: 295–338.
18. Board RG. The course of microbial infection of the hens' egg. J Appl Bacteriol 1966; **29**: 319–41.
19. Board RG. The growth of Gram negative bacteria in the hens' egg. J Appl Bacteriol 1964; **27**: 350–64.
20. Board RG. Bacterial growth on, and penetration of the shell membranes of the hens' egg. J Appl Bacteriol 1965; **1**: 197–205.
21. Anon. Update on *Salmonella* infection. PHLS–SVS 1991.
22. Hinton M, Threlfall EJ, Rowe B. The invasive potential of *Salmonella enteritidis* phage type 4 for young chicks. Lett Appl Microbiol 1990; **10**: 237–9.
23. Rozak DB, Grimes DJ, Colwell RR. Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. Can J Microbiol 1984; **30**: 334–8.
24. Humphrey TJ, Richardson NP, Gawler AHL, Allen MJ. Heat resistance of *Salmonella enteritidis* PT 4: the influence of prior exposure to alkaline conditions. Lett Appl Microbiol 1991; **12**: 258–60.

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Observations on the mode of bacterial infection of hens' eggs

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1. SUMMARY

Processes involved in the bacterial infection of eggs are reviewed and a simple technique to aid studies of the infection is described.

2. INTRODUCTION

There have been two major phases in the studies of microbial infection of hens' eggs. The first (in the years 1930–1960) sought to identify factors that caused the addling of stored eggs [1]. The second, which began in the mid 1980s, is concerned with infection of eggs with *Salmonella enteritidis*. This contribution summarizes the major observations made in these studies and describes a technique for future work.

3. INFECTION OF EGGS

Few, if any, eggs contain microorganisms at oviposition [1]. When eggs are infected during formation the contaminants come from the ovary (trans-ovarian infection) or the oviduct (oviducal infection) [2]. In the 1930s ovarian infection with *Salmonella pullorum* or *S. gallinarum* caused vertical transmission of disease in poultry flocks worldwide [3]. The current pandemic [4] of human salmonellosis associated with infection of eggs and egg products with *S. enteritidis* has revived interest in infection in vivo. Humphrey et al. [5] recorded an incidence of about 0.6% infection of the contents, mainly in the albumen, of upwards of 5700 eggs produced by laying flocks known to have been infected with *S. enteritidis*.

Translocation of bacteria across the shell (trans-shell infection [2]) is a key stage in the addling of eggs [6]. Water, and a temperature difference between it and the egg, is a common cause of translocation since the contraction of a warm egg in cold water sucks bacteria through the pores in the shell. The infection is confined to the shell membranes for 10–20 days in eggs stored at ambient temperature (e.g. [1]). Confinement is followed by the sudden onset of multiplication resulting in gross contamination and macroscopic changes of egg contents infected with rot-producing bacteria [6]. This sequence of events also occurs in eggs deliberately infected with *S. enteri-*

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tidis [7], but there are no changes in the appearance of the white or yolk.

The mechanisms that trigger the sudden onset of bacterial multiplication have not been defined precisely. Gillespie and Scott [1] concluded that "the bacteria may begin growth, slowly attack the (shell) membranes, and eventually reach the white. Once infection has been established in this way, subsequent development to a bacterial rot takes place relatively quickly". This latter contention is untenable in view of the many studies that have shown that the albumen is a very unfavourable medium for microbial growth [8,9]. Brooks [10] contended that a change in the shell membranes rendered these susceptible to bacterial attack — thereby triggering the generalized infection — and that such a change "becomes more rapid on about the 12th day" of storage of eggs at room temperature. He found that storage had no demonstrable effect on the ability of albumen to support microbial growth. Indeed he concluded that the sudden onset of a generalized infection could not be attributed to diffusion of nutrients from the yolk to the white. Recently it was speculated [5] that such diffusion accounted for the onset of growth of *S. enteritidis* in egg contents. In practice, little attention has been given to the exchange of materials other than water between the white and yolk of stored eggs.

Board [6] associated the sudden onset of a generalized infection of the contents of eggs with rot-producing bacteria with changes in the physical structure of the albumen and the density of the yolk. Loss of viscosity and breakdown of the fibrous network of the albuminous sac lead to a progressive increase in the mobility of the yolk. With storage the yolk absorbs water, becomes less dense, floats upwards and makes contact with the shell membranes. Such events result in an albumen-depleted niche in which organisms grow and initiate a generalized infection of the contents of eggs stored at ambient temperature. Subsequent studies [11] with eggs infected with pseudomonads and stored at 4°C did not support this scenario. Gross infection of the albumen appeared to follow the growth of pseudomonads that had made contact with the yolk before the latter had reached the shell membranes. This interpretation was examined in the present study.

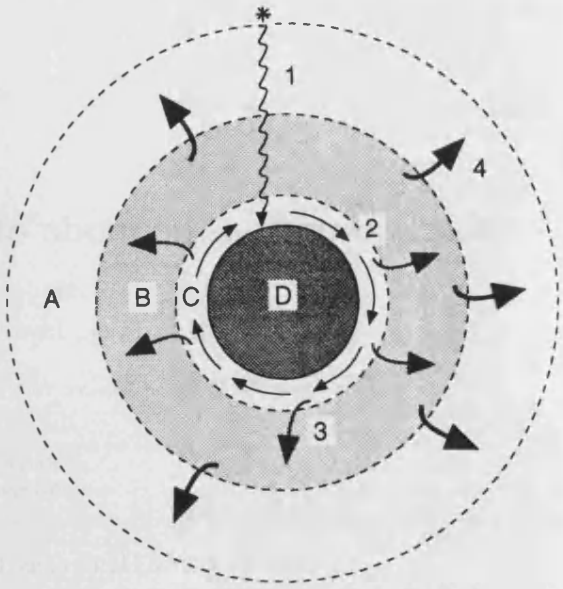

Fig. 1. Sequence of events leading to generalized infection of egg contents with *Pseudomonas putida*. Contents of a freshly laid egg were poured into a square (10×10 cm) Petri dish and inoculated in the outer thin albumen (asterisk) with pseudomonads in a plug of water agar. The numbered arrows refer to steps in the infection process (see text for details). A, outer thin white; B, albuminous sac; C, inner thin white; D, yolk.

The shells of freshly laid eggs were wiped with 70% (v/v) ethanol, broken and the contents poured gently into a square (10 × 10 cm) Petri dish. *Pseudomonas putida* in a plug of water agar (1.0% w/v) was placed in the thin albumen at the edge of the dish (Fig. 1). The dish was incubated at 4 or 20°C and examined frequently under UV light (350 nm). The following sequence was noted: (1) pseudomonads migrated from the water agar (sic the shell membranes) into the albumen; some progressed as far as the surface of the yolk (Fig. 1); (2) those that made contact with the yolk multiplied and, as judged by the occurrence of fluorescent pigment, remained in a confined zone for a short while before spreading throughout the inner thin white; (3) organisms spread outwards from the inner thin white into the albuminous sac which became fluorescent; (4) finally, infection spread to the outer thin white and the entire

albumen fluoresced. Analogous results were obtained at 4 and 20°C.

These observations demonstrate that the sudden development of gross infection of an egg's contents can be triggered by bacteria that make contact with the surface of the yolk. They also raise questions about the possible role of the diffusion of nutrients from the yolk and an organism's production of siderophores in the infection process. We have evidence (unpublished) that salmonellae are attracted towards the yolk. The possible involvement of chemotaxis and the occurrence of a gradient in nutrients from the yolk outwards raises the question: does diffusion establish such a gradient? Garibaldi [12] claimed that organisms formed siderophores when deprived of iron through the sequestration of this element by ovotransferrin. This view gained no support from the work of Tranter and Board [8]. The present study has raised this question yet again but in a different context. A single fluorescent green patch populated with actively motile pseudomonads has been observed in the albumen taken from eggs, the shell membranes of which were deliberately infected with the test organism [1,6]. We have seen such patches on many occasions in eggs infected in this manner but have been unable to determine the actual location of the spot in an egg prior to destructive sampling. The present study has demonstrated its association with the yolk. We speculate that the yolk supplies the nutrients, particularly iron, required for the formation of this patch of organisms. Once established, the organisms obtain iron from that present in the albumen through siderophore synthesis, such synthesis occurring at the leading edge of growth. The simple technique described in this article ought to allow investigation of these issues.

REFERENCES

[1] Gillespie, J.M. and Scott, W.J. (1950) Aust. J. Appl. Sci. 1, 514–530.
[2] Duguid, J.P. and North, R.A.E. (1991) J. Med. Microbiol. 34, 65–72.
[3] Alder, H.E. (1965) Food Technol. 623, 191–201.
[4] Rodrigue, D.C., Tauxe, R.V. and Rowe, B. (1990) Epidemiol. Infect. 105, 21–27.
[5] Humphrey, T.J., Whitehead, A., Gawler, A.H.L., Henley, A. and Rowe, B. (1991) Epidemiol. Infect. 106, 489–496.
[6] Board, R.G. (1966) J. Appl. Bact. 29, 319–341.
[7] Clay, C.E. and Board, R.G. (1991) Epidemiol. Infect. 106, 271–281.
[8] Tranter, H.S. and Board, R.G. (1984) J. Appl. Bact. 56, 53–61.
[9] Lock, J.L. and Board, R.G. (1992) Epidemiol. Infect. 108, 389–396.
[10] Brooks, J. (1960) J. Appl. Bact. 23, 499–509.
[11] Board, R.G. and Ayres, J.C. (1965) Appl. Microbiol. 13, 358–364.
[12] Garibaldi, J.A. (1970) Appl. Microbiol. 20, 558–560.